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DATE: Thursday, June 10, 2004

Hide?	Set Name	Query	Hit Count
		DB=PGPB; PLUR=YES; OP=AND	
☐	L1	FITZGERALD.IN.	261
☐	L2	L1 and domain	29
☐	L3	(pe or pea or enterotoxin or entero-toxin or pseudomonas or pelike or pe-like).clm. and (chimeria or chimera or chimaera or chimaeria or recombinant or fusion or multidomain or multi-domain or fused or linked or joined or combination or complex or engineered or engineering).clm.	1001
☐	L4	L3 and pseudomonas	562
☐	L5	L4 and (disulfide or di-sulfide or loop or carrier or cysteine-cysteine or cys-cys or c-c or cyscys)	456
☐	L6	L4 and (disulfide or di-sulfide or loop or carrier or cysteine-cysteine or cys-cys or cyscys)	452
☐	L7	L4 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys)	270
☐	L8	L4 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys).clm.	23
		DB=USPT; PLUR=YES; OP=AND	
☐	L9	(pe or pea or enterotoxin or entero-toxin or pseudomonas or pelike or pe-like).clm. and (chimeria or chimera or chimaera or chimaeria or recombinant or fusion or multidomain or multi-domain or fused or linked or joined or combination or complex or engineered or engineering).clm.	1242
☐	L10	L9 and pseudomonas	685
☐	L11	L10 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys).clm.	25
☐	L12	('6423513' |'6492498' |'5942602' |'5980895' |'5965406' |'6022950')!.PN.	6
		DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND	
☐	L13	pe-like	34

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Hide?	Set Name	Query	Hit Count
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☐	L1	FITZGERALD.IN.	261
☐	L2	L1 and domain	29
☐	L3	(pe or pea or enterotoxin or entero-toxin or pseudomonas or pelike or pe-like).clm. and (chimeria or chimera or chimaera or chimaeria or recombinant or fusion or multidomain or multi-domain or fused or linked or joined or combination or complex or engineered or engineering).clm.	1001
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☐	L6	L4 and (disulfide or di-sulfide or loop or carrier or cysteine-cysteine or cys-cys or cyscys)	452
☐	L7	L4 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys)	270
☐	L8	L4 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys).clm.	23
		DB=USPT; PLUR=YES; OP=AND	
☐	L9	(pe or pea or enterotoxin or entero-toxin or pseudomonas or pelike or pe-like).clm. and (chimeria or chimera or chimaera or chimaeria or recombinant or fusion or multidomain or multi-domain or fused or linked or joined or combination or complex or engineered or engineering).clm.	1242
☐	L10	L9 and pseudomonas	685
☐	L11	L10 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys).clm.	25
☐	L12	('6423513' |'6492498' |'5942602' |'5980895' |'5965406' |'6022950')!.PN.	6
		DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND	
☐	L13	pe-like	34

END OF SEARCH HISTORY

WEST Search History

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DATE: Thursday, June 10, 2004

Hide?	Set Name	Query	Hit Count
		DB=PGPB; PLUR=YES; OP=AND	
☐	L1	FITZGERALD.IN.	261
☐	L2	L1 and domain	29
☐	L3	(pe or pea or enterotoxin or entero-toxin or pseudomonas or pelike or pe-like).clm. and (chimeria or chimera or chimaera or chimaeria or recombinant or fusion or multidomain or multi-domain or fused or linked or joined or combination or complex or engineered or engineering).clm.	1001
☐	L4	L3 and pseudomonas	562
☐	L5	L4 and (disulfide or di-sulfide or loop or carrier or cysteine-cysteine or cys-cys or c-c or cyscys)	456
☐	L6	L4 and (disulfide or di-sulfide or loop or carrier or cysteine-cysteine or cys-cys or cyscys)	452
☐	L7	L4 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys)	270
☐	L8	L4 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys).clm.	23
		DB=USPT; PLUR=YES; OP=AND	
☐	L9	(pe or pea or enterotoxin or entero-toxin or pseudomonas or pelike or pe-like).clm. and (chimeria or chimera or chimaera or chimaeria or recombinant or fusion or multidomain or multi-domain or fused or linked or joined or combination or complex or engineered or engineering).clm.	1242
☐	L10	L9 and pseudomonas	685
☐	L11	L10 and (disulfide or di-sulfide or loop or cysteine-cysteine or cys-cys or cyscys).clm.	25
☐	L12	('6423513' |'6492498' |'5942602' |'5980895' |'5965406' |'6022950')!.PN.	6

END OF SEARCH HISTORY

Search Results - Record(s) 1 through 6 of 6 returned.

L12: Entry 1 of 6 File: USPT Dec 10, 2002

US-PAT-NO: 6492498
DOCUMENT-IDENTIFIER: US 6492498 B1
**** See image for Certificate of Correction ****

TITLE: Multimeric immunotoxins

DATE-ISSUED: December 10, 2002

US-CL-CURRENT: 530/391.7; 424/183.1, 530/300, 530/350, 530/387.1

INT-CL: [07] C07 K 16/00

L12: Entry 2 of 6 File: USPT Jul 23, 2002

US-PAT-NO: 6423513
DOCUMENT-IDENTIFIER: US 6423513 B1

TITLE: Polynucleotides encoding protease-activatable pseudomonas exotoxin a-like proproteins

DATE-ISSUED: July 23, 2002

US-CL-CURRENT: 435/71.3; 424/183.1, 424/184.1, 424/192.1, 424/193.1, 424/236.1, 424/260.1, 435/252.33, 435/320.1, 435/69.1, 435/69.3, 435/69.7, 435/71.1, 530/351, 530/356, 530/387.3, 530/391.7

INT-CL: [07] C12 P 21/04, C12 P 21/06, C12 N 15/09, A61 K 39/00

L12: Entry 3 of 6 File: USPT Feb 8, 2000

US-PAT-NO: 6022950
DOCUMENT-IDENTIFIER: US 6022950 A
**** See image for Certificate of Correction ****

TITLE: Hybrid molecules having translocation region and cell-binding region

DATE-ISSUED: February 8, 2000

US-CL-CURRENT: 530/350; 530/351, 530/387.3, 530/388.1

INT-CL: [06] C07 K 14/00, C07 K 14/485, C07 K 14/55, C07 K 16/28

L12: Entry 4 of 6 File: USPT Nov 9, 1999

US-PAT-NO: 5980895
DOCUMENT-IDENTIFIER: US 5980895 A

TITLE: Immunotoxin containing a disulfide-stabilized antibody fragment joined to a

Pseudomonas exotoxin that does not require proteolytic activation

DATE-ISSUED: November 9, 1999

US-CL-CURRENT: 424/178.1; 424/236.1, 530/387.3, 530/387.7

INT-CL: [06] A61 K 39/395, C07 K 16/00

L12: Entry 5 of 6 File: USPT Oct 12, 1999

US-PAT-NO: 5965406
DOCUMENT-IDENTIFIER: US 5965406 A
**** See image for Certificate of Correction ****

TITLE: Recombinant DNAS encoding three-part hybrid proteins

DATE-ISSUED: October 12, 1999

US-CL-CURRENT: 435/69.7; 435/252.33, 435/320.1, 536/23.4

INT-CL: [06] C12 N 1/21, C12 N 15/12, C12 N 15/63, C12 P 21/02

L12: Entry 6 of 6 File: USPT Aug 24, 1999

US-PAT-NO: 5942602
DOCUMENT-IDENTIFIER: US 5942602 A

TITLE: Growth factor receptor antibodies

DATE-ISSUED: August 24, 1999

US-CL-CURRENT: 530/388.22; 424/1.49, 424/178.1, 424/9.34, 530/387.3, 530/388.8, 530/388.85, 530/391.3, 530/391.7, 536/23.1, 536/24.31

INT-CL: [06] C07 K 16/28, C07 K 16/30

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☐ 10. 20040008724. 02 May 03. 15 Jan 04. Communications switching architecture. Devine, Geoffrey, et al. 370/466; 370/493 H04J003/16.

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1. 20030054012. 12 May 00. 20 Mar 03. PSEUDOMONAS EXOTOXIN A-LIKE CHIMERIC IMMUNOGENS FOR ELICITING A SECRETORY IGA-MEDIATED IMMUNE RESPONSE. FITZGERALD, DAVID J., et al. 424/190.1; 435/5 A61K039/02 C12Q001/70.

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A61K039/108 A61K047/48 A61P035/00 C07K014/21 C07K016/30 C07K019/00 C12N015/09 C12N015/31 C12N015/62 C12P021/04 C12P021/06.

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☐ 34. 1475600. 27 Nov 23. Hand seed sower. MAX SCHLING. 222/544; 222/162 222/566.

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☑	WO009820135A2	all	all	N/A	PGPB,USPT,USOC,EPAB,JPAB,DWPI
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Search Results - Record(s) 1 through 34 of 34 returned.

☐ 1. Document ID: US 20030054012 A1

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L13: Entry 1 of 34 File: PGPB Mar 20, 2003

PGPUB-DOCUMENT-NUMBER: 20030054012
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030054012 A1

TITLE: PSEUDOMONAS EXOTOXIN A-LIKE CHIMERIC IMMUNOGENS FOR ELICITING A SECRETORY IGA-MEDIATED IMMUNE RESPONSE

PUBLICATION-DATE: March 20, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
FITZGERALD, DAVID J.	ROCKVILLE	MD	US	
MRSNY, RANDALL J.	REDWOOD CITY	CA	US	

US-CL-CURRENT: 424/190.1; 435/5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

☐ 2. Document ID: US 6426075 B1

L13: Entry 2 of 34 File: USPT Jul 30, 2002

DOCUMENT-IDENTIFIER: US 6426075 B1
TITLE: Protease-activatable pseudomonas exotoxin A-like proproteins

Abstract Text (1):
This invention provides protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins. The proproteins comprise (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence

substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence. The invention also provides methods of using these proproteins for killing target cells.

Brief Summary Text (10):
Pseudomonas Exotoxin A ("PE") is translocated into the cytosol after a furin recognition site in domain II is cleaved by furin. Protease-activatable PE-like proproteins are engineered to replace the furin recognition site by a site recognized by a protease made or secreted by a cell targeted for death, for example, a cancer cell. Upon cleavage by the target protease, the PE-like proprotein is translocated into the cytosol where the toxin's ADP-ribosylating activity kills the cell by interfering with polypeptide elongation.

Brief Summary Text (11):
The PE-like proproteins of this invention offer several advantages. First, because they are activated by a target protease, and not by furin, their toxicity is significantly more cell-specific than native PE. Second, when the cysteine-cysteine loop of PE domain II is cleaved, the disulfide bond, before it is reduced, holds the cell-recognition domain attached to the rest of PE. Many cancer cells secrete cell-specific proteases that tend to accumulate around the cell. For example, prostate cancer cells secrete prostate specific antigen. Therefore, the proproteins of this invention may be cleaved before entering the target-cell. However, the protease activatable sequence is introduced into the cysteine-cysteine loop of a domain II-like sequence of the proprotein. Therefore, the cell recognition domain is still attached upon cleavage of the proprotein outside the cell, and still is available to bind to a cell surface receptor for subsequent endocytosis. Third, by selecting a proper cell recognition domain, the toxins can be targeted to bind to specific cell types. For example, the modified PE proprotein can be administered as an immunotoxin to further increase its selective toxicity to the desired cells.

Brief Summary Text (14):
In one aspect this invention provides a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface-receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence.

Brief Summary Text (15):
In one embodiment of a PE-like proprotein, the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO:1) modified with amino acids substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280. In another embodiment the protease activatable sequence is cleavable by a protease secreted by a cancer cell. In another embodiment, the cell recognition domain comprises an antibody that specifically binds to a cancer cell surface marker. In another embodiment, the protease activatable sequence is cleavable by prostate specific antigen ("PSA"), urokinase, neutral endoprotease, stromelysin, collagenase, cathepsin B, or cathepsin D. In another embodiment, the PE Ib-like domain, the cytotoxicity domain and the ER retention. sequence together have the sequence of domains Ib and III of native PE. In other embodiment, the cell recognition domain is coupled to the modified translocation domain through a peptide bond (i.e., as a fusion protein) or through a chemical linkage.

Brief Summary Text (16):
In another aspect, this invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention.

Brief Summary Text (17):
In another aspect, this invention provides a recombinant polynucleotide comprising a nucleotide sequence encoding a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the recombinant polynucleotide is an expression vector further comprising an expression control sequence operatively linked to the nucleotide sequence.

Brief Summary Text (18):
In another aspect, this invention provides a method for killing a target cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the cancer cell is, without limitation, a prostate cancer cell, a breast cancer cell or a colon cancer cell.

Brief Summary Text (19):
In another aspect, this invention provides a method for therapeutically treating a subject suffering from cancer comprising administering to the subject a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. More specifically, the PE-like proprotein comprises a protease activatable sequence that is cleavable by an enzyme produced by the cancer cell. The PE-like proprotein can be administered as a pharmaceutical composition.

Detailed Description Text (5):
As used herein, "activates" includes reference to the formation of a cytotoxic PE-like molecule having an IC50 of no more than 30 ng/ml according to cytotoxicity assays as provided, for example, in the Examples. See also, Brinkmann et al., Proc. Natl. Acad. Sci. USA, 88:8616-8620 (1991).

Detailed Description Text (50):
Protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins are polypeptides having structural domains organized, except as provided herein, in the same general sequence as the four structural domains of PE, and having certain functions (e.g., cell recognition, cytosolic translocation, cytotoxicity and endoplasmic reticulum retention) also possessed by the functional domains of P.E. More specifically, the general order is: domain Ia, domain II, domain Ib, domain III. However, as described in more detail herein, domain Ia can be eliminated and replaced by a binding protein chemically coupled to the molecule, or, a cell recognition domain can be inserted just before the ER retention sequence in domain III. Domain Ib can be eliminated. Domain II is positioned to the amino-terminal side of domain III.

Detailed Description Text (51):
In contrast to native PE, the PE-like proproteins of this invention are engineered to eliminate the native furin cleavage site of domain II and to include within domain II of PE (and, preferentially, within a cysteine-cysteine loop of domain II) a protease activatable sequence that is cleavable by a target protease. Preferably, the target protease is a protease produced by a cell targeted for death, e.g., a cancer cell.

Detailed Description Text (52):
Accordingly, PE-like proproteins include the following structural domains comprised of amino acid sequences, the domains imparting particular functions to the proprotein: (1) a "cell recognition domain" that functions as a ligand for a cell

surface receptor and that mediates binding of the protein to a cell; (2) a "translocation domain" that mediates translocation from the endosomes to the cytosol cytosol and that includes the protease-activatable sequence and is substantially unactivatable by furin; (3) an optional "PE 1b-like domain" of up to 1500 amino acids; (4) a "cytotoxic domain" that functions to kill cells, preferably, by interfering with ADP-ribosylation activity; and (5) an "endoplasmic reticulum ("ER") ("ER") retention sequence" that functions to translocate the molecule from the endosome to the endoplasmic reticulum, from which it enters the cytosol.

Detailed Description Text (53):
The relationship of PE structure to its function has been extensively studied. The amino acid sequence of PE has been re-engineered to provide new functions, and many amino acids or peptide segments critical and non-critical to PE function have been identified. Accordingly, the PE-like proproteins of this invention can incorporate these structural modifications to PE within the boundaries set forth herein.

Detailed Description Text (60):
In preferred embodiments, the PE-like proproteins of the present invention can be modified with a cell specific ligand to target the PE-like proproteins to particular cells. PE-like proproteins so modified preferentially bind to cells displaying the binding partner to that ligand. In a preferred embodiment, the cell specific ligand is an antibody; thereby, the proprotein is an immunotoxin. Cell specific ligands which are proteins can often be formed in part or in whole as a fusion protein with the PE-like proproteins of the present invention. A "fusion protein" includes reference to a polypeptide formed by the joining of two or more polypeptides through a peptide bond formed by the amino terminus of one polypeptide and the carboxyl terminus of the other polypeptide. The fusion protein may be formed by the chemical coupling of the constituent polypeptides but is typically expressed as a single polypeptide from a nucleic acid sequence encoding the single contiguous fusion protein. Included among such fusion proteins are single chain Fv fragments (scFv). Particularly preferred targeted PE-like proproteins are disulfide stabilized immunotoxins which can be formed in part as a fusion protein as exemplified herein. Other protein cell specific ligands can be formed as fusion proteins using cloning methodologies well known to the skilled artisan.

Detailed Description Text (62):
In one embodiment, protease-activatable PE-like proproteins are immunotoxins directed against particular cancer cells. Accordingly, the cell recognition domain is an antibody that specifically binds to a cell surface receptor or marker specific for a cancer cell. This includes, for example, prostate cancer cells, breast cancer cells or colon cancer cells. In alternative embodiments, the proprotein can be a fusion protein or a conjugate protein. In the fusion protein, domain Ia is replaced with a polypeptide sequence for an immunoglobulin heavy chain from an immunoglobulin specific for the cancer cell. The light chain of the immunoglobulin can be co-expressed with the proprotein so as to form a light chain-heavy chain dimer. In the conjugate protein, the antibody is chemically linked to a polypeptide comprising the other domains of the proprotein.

Detailed Description Text (63):
The procedure for attaching a PE-like proprotein to an antibody or other cell specific ligand will vary according to the chemical structure of the toxin. Antibodies contain a variety of functional groups; e.g., sulfhydryl (--S), carboxylic acid (COOH) or free amine (--NH.sub.2) groups, which are available for reaction with a suitable functional group on a toxin. Additionally, or alternatively, the antibody or PE-like proprotein can be derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Ill.

Detailed Description Text (64):

A bifunctional linker having one functional group reactive with a group on the PE-like proprotein, and another group reactive with a cell specific ligand, can be used used to form a desired conjugate. Alternatively, derivatization may involve chemical treatment of the PE-like proprotein or the cell specific ligand, e.g., glycol cleavage of the sugar moiety of a glycoprotein antibody with periodate to generate free aldehyde groups. The free aldehyde groups on the antibody may be reacted with free amine or hydrazine groups on the antibody to bind the PE-like proprotein thereto. (See U.S. Pat. No. 4,671,958). Procedures for generation of free sulfhydryl groups on antibodies or other proteins, are also known (See U.S. Pat. No. 4,659,839).

Detailed Description Text (66):
In some circumstances, it is desirable to free the PE-like proprotein from the antibody or other cell specific ligand when the conjugate has reached its target site. Therefore, conjugates comprising linkages which are cleavable in the vicinity or within the target site may be used when the toxin is to be released at the target site. Cleaving of the linkage to release the agent from the ligand may be prompted by enzymatic activity or conditions to which the immunoconjugate is subjected either inside the target cell or in the vicinity of the target site. A number of different cleavable linkers are known to those of skill in the art. See U.S. Pat. Nos. 4,618,492; 4,542,225, and 4,625,014. SPDP is a reversible NHS-ester, pyridyl disulfide cross-linker used to conjugate amine-containing molecules to sulfhydryls. Another chemical modification reagent is 2-iminothiolane which reacts with amines and yields a sulfhydryl. Water soluble SPDP analogs, such as Sulfo-LC-SPDP (Pierce, Rockford, Ill.) are also available. SMPT is a reversible NHS-ester, pyridyl disulfide cross-linker developed to avoid cleavage in vivo prior to reaching the antigenic target. Additionally, the NHS-ester of SMPT is relatively stable in aqueous solutions.

Detailed Description Text (67):
C. PE-Like Translocation Domain

Detailed Description Text (68):
Protease-activatable PE-like proproteins also comprise an amino acid sequence encoding a "modified PE translocation domain." The modified PE translocation domain includes a cysteine-cysteine loop that includes a protease activatable sequence, and is engineered to eliminate the native furin recognition site. It comprises an amino acid sequence sufficient to effect translocation of chimeric proteins that have been endocytosed by the cell into the cytosol. A proprotein that does not contain the sequence RXXR (SEQ ID NO:30) with in the cysteine-cysteine loop is substantially un-activatable by furin and, consequently, substantially non-toxic to cells not having an enzyme that cleaves the protease-activatable sequence. The amino acid sequence generally is substantially identical to a sequence selected from domain II of PE. Upon cleavage by a protease that recognizes the protease-activatable sequence and reduction of the disulfide bond, the proprotein is now activated, i.e., is enabled for translocation into the cytosol and subsequent cytotoxic activity.

Detailed Description Text (69):
More specifically, protease-activatable PE-like proproteins comprise a cysteine-cysteine loop that comprises the protease activatable sequence. The loop generally has between about 10 and 50 amino acids, more preferably, about 23 amino acids, as in native PE. The protease activatable sequence can be located anywhere within the cysteine-cysteine loop. Preferably, the sequence is more than 5 amino acids from the cysteine residues involved in the disulfide bond. More preferably, the cysteine-cysteine loop has the sequence of the loop in native PE except for substitutions that introduce the protease activatable sequence. The cysteine-cysteine loop also is modified to eliminate the native furin recognition sequence. Furin recognizes the sequence RXXR (SEQ ID NO:30), wherein X is any amino acid. This sequence occurs in native PE at amino acids 276-279.

Detailed Description Text (75):
Amino acid sequences that can serve as protease activatable sequences are chosen from peptide substrates of the desired protease. Proteases and their amino acid sequence substrates are well known in the art. For example, urokinase is an enzyme found in many metastatically active cancers. The sequence of urokinase is described by Nagai et al., Gene, 36:183-188 (1985); Riccio et al. Nucl. Acids Res., 13:2759-2771 (1985); and, Holmes et al., Bio/Technology 3:923-929 (1985), all of which are incorporated herein by reference. Peptide substrates for urokinase ("urokinase activatable sequences) include the sequences: DR/VYIHPF (SEQ ID NO:3) from angiotensin, VVCGER JGFFYTP (SEQ ID NO:4) from the insulin B chain, FFYTPK/A (SEQ ID NO:5), and from adrenal corticotrophic hormone (ACTH) the sequences: KRRPVK/VYP (SEQ ID NO:6), PVGKKR/RPVKVY (SEQ ID NO:7), KPVGKK/RRPVKV (SEQ ID NO:8), and GKPVGK/KRRPVK (SEQ ID NO:9), where "/" indicates the cleavage site. In particularly preferred embodiments, the urokinase activatable sequence has the sequence TFAGNAVRRISVGQ (SEQ ID NO:10). Generally, SEQ ID NO:10 is inserted between residues 271 and 283 of the PE-like proprotein. The sequences of the urokinase activatable sequences disclosed above can be shortened by one or two amino acid residues at the carboxyl and/or amino terminal ends such that at least two residues of the prototype sequences are maintained on either side of the cleavage site.

Detailed Description Text (76):
In other embodiments, the protease activatable sequence is derived from any substrate of prostate-specific antigen ("PSA"). This includes, for example, semenogelin I or semenogelin II, or insulin-like growth factor binding protein. A preferred protease activatable sequence is derived from semenogelin I. Protease activatable sequences from semenogelin I (PSA activatable sequences) include: sem 1 having the sequence SKGSFSIQY/TYHV (SEQ ID NO:11), sem 3 having the sequence HLGGSQQLL/HNKQ (SEQ ID NO:12), and sem 5 having the sequence SKGKGTSSQY/SNTE (SEQ ID NO:13), where "/" indicates the cleavage at position 279. In particularly preferred embodiments the sem 1, sem 3, and sem 5 protease activatable sequences are substituted for the native PE-like proprotein amino acids residing between residues 271 and 283. The sequences of the PSA activatable sequences disclosed above can be shortened by one or two amino acid residues at the carboxyl and/or amino terminal ends such that at least two residues of the prototype sequences are maintained on either side of the cleavage site.

Detailed Description Text (81):
Protease activatable PE-like proproteins optionally include an amino acid sequence encoding a "PE 1b-like domain." The PE 1b-like domain is located at the native 1b domain location of PE, between the translocation domain (e.g., domain II) and the cytotoxic domain (e.g., domain III). The PE 1b-like domain can be up to about 1500 amino acids. This includes domains having between about 15 amino acids and about 350 amino acids or about 15 amino acids and about 50 amino acids.

Detailed Description Text (84):
PE-like proproteins also comprise an amino acid sequence encoding a "cytotoxicity domain" and an "endoplasmic reticulum (`ER`) retention sequence." The cytotoxicity domain has a sequence sufficiently complementary to domain III of native PE to confer ADP-ribosylating activity, thereby rendering the construct cytotoxic. The ER retention sequence functions in translocating the proprotein to from the endosome to the endoplasmic reticulum, from where it is transported to the cytosol. The cytotoxic domain is located at the position of domain III in PE.

Detailed Description Text (90):
F. Methods of Making PE-like Proproteins

Detailed Description Text (91):
PE-like proproteins preferably are produced recombinantly, as described below. This invention also envisions the production of PE chimeric proteins by chemical

synthesis using methods available to the art.

Detailed Description Text (92):
G. Testing PE-like Proproteins

Detailed Description Text (93):
Having selected various structures as domains of the proprotein, the function of these domains, and of the proprotein as a whole, can be tested to detect functionality. PE-like proproteins can be tested for cell recognition, cleavability, cleavability, cytosolic translocation and cytotoxicity using routine assays. The entire proprotein protein can be tested, or, the function of various domains can be tested by substituting them for native domains of the wild-type toxin.

Detailed Description Text (106):
In another method, the ability of the translocation domain and ER retention sequence to effect translocation to the cytosol can be tested with a construct containing a domain III having ADP ribosylation activity. Briefly, cells are seeded in tissue culture plates and exposed to the PE-like proprotein or to an engineered PE exotoxin containing the modified translocation domain or ER retention sequence in place of the native domains. ADP ribosylation activity is determined as a function of inhibition of protein synthesis by, e.g., monitoring the incorporation of .sup.3 H-leucine.

Detailed Description Text (107):
III. Recombinant Polynucleotides Encoding PE-Like Proproteins

Detailed Description Text (110):
With the protease activatable sequences herein provided, and the PE sequence as disclosed in SEQ ID NO:1, one of skill can readily construct a variety of clones containing functionally equivalent nucleic acids, such as nucleic acids which differ in sequence but which encode the same PE-like proprotein. PE-like proprotein related compositions such as PE-like proprotein fusion proteins comprising a cell-specific ligand can also be constructed. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art and exemplified herein. Other examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or Current Protocols in Molecular Biology, (Ausubel, et al. (eds.), Greene Publishing and Wiley-Interscience, New York (1987).

Detailed Description Text (112):
Polynucleotides encoding PE-like proproteins or subsequences thereof, such as a protease activatable sequence, can be prepared by any suitable method including, for example, cloning and restriction of appropriate sequences as discussed supra, or by direct chemical synthesis by methods such as the phosphotriester method of Narang et al. Meth. Enzymol. 68: 90-99 (1979); the phosphodiester method of Brown et al., Meth. Enzymol. 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage et al., Tetra. Lett., 22: 1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Letts., 22(20): 1859-1862, e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168; and, the solid support method of U.S. Pat. No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Detailed Description Text (113):
Nucleic acids encoding native PE exotoxin can be modified to form the PE-like proproteins of -the present invention. Modification by site-directed mutagenesis is well known in the art. Native PE exotoxin nucleic acids can be amplified by in vitro methods. Amplification methods include the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (SSR). A wide variety of cloning methods, host cells, and in vitro amplification methodologies are well-known to persons of skill.

Detailed Description Text (115):
Once the nucleic acids encoding an PE-like proprotein of the present invention is isolated and cloned, one may express the desired protein in a recombinantly engineered cell such as bacteria, yeast, insect and mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of proteins. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made. In brief, the expression of natural or synthetic nucleic acids encoding the isolated proteins of the invention will typically be achieved by operably linking the DNA or cDNA to an expression control sequence (e.g., a promoter which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding the protein. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator.

Detailed Description Text (116):
One of skill would recognize that modifications can be made to a nucleic acid encoding a PE-like proprotein without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

Detailed Description Text (119):
Nucleic acids encoding PE-like proproteins of the present invention may be expressed expressed in a variety of host cells, including E. coli, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For E. coli this includes a promoter such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, etc., and a polyadenylation sequence, and may include splice donor and acceptor sequences. The plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for E. coli and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

Detailed Description Text (120):
Once expressed, the recombinant fusion proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity

columns, column chromatography, gel electrophoresis and the like (see, generally, R. R. Scopes, Protein Purification, Springer-Verlag, N.Y. (1982)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or or more homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the PE-like proproteins of the present invention may then be used therapeutically.

Detailed Description Text (121):
The PE-like proproteins of the present invention can also be constructed in whole or or in part using standard synthetic methods. Solid phase synthesis of isolated proteins of the present invention of less than about 50 amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany and Merrifield, Solid-Phase Peptide Synthesis; pp. 3-284 in The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A., Merrifield, et al. J. Am. Chem. Soc., 85: 2149-2156 (1963), and Stewart et al., Solid Phase Peptide Synthesis, 2nd ed. Pierce Chem. Co., Rockford, Ill. (1984). Proteins of greater length may be synthesized by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N, N'-dicycylohexylcarbodiimide)) is known to those of skill.

Detailed Description Text (122):
Eliminating nucleotides encoding amino acids 1-252 yields a construct referred to as "PE40." Eliminating nucleotides encoding amino acids 1-279 yields a construct referred to as "PE37." (See Pastan et al., U.S. Pat. No. 5,602,095.) The practitioner can ligate sequences encoding cell recognition domains to the 5'end of these platforms to engineer PE-like chimeric proteins that are directed to particular cell surface receptors. These constructs optionally can encode an amino-terminal methionine. A cell recognition domain can be inserted into such constructs in the nucleotide sequence encoding the ER retention sequence.

Detailed Description Text (125):
In preferred embodiment, the PE-like proproteins of the present invention are attached to antibodies to form immunotoxins. Particularly preferred are disulfide stabilized antibodies as exemplified herein. The attachment may be by covalent or non-covalent means (e.g., biotin and avidin). Typically, covalent attachment can be accomplished by construction of fusion proteins or by the use of chemical linkers as discussed, supra. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

Detailed Description Text (139):
In another embodiment, this invention provides for fully human antibodies which serve as cell specific ligands for construction of PE-like proproteins. Human antibodies consist entirely of characteristically human polypeptide sequences. The human antibodies of this invention can be produced in using a wide variety of methods (see, e.g., Larrick et al., U.S. Pat. No. 5,001,065, for review).

Detailed Description Text (143):
The present invention provides nucleic acids encoding proteases for cleavage of the desired protease activatable sequence (protease nucleic acids). The mammalian cells can be altered to express the cognate protease to a particular protease activatable sequence. Thus, mammalian cells can be altered for susceptibility to a particular PE-like proprotein.

Detailed Description Text (144):
The present invention also provides nucleic acids encoding the PE-like proprotein compositions of the present invention (PE-like proprotein nucleic acids). These PE-

like proproteins can be used to inhibit protein synthesis in recombinant or native cells expressing the cognate protease.

Detailed Description Text (150):
Ex vivo mammalian cell transfection for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject mammalian organism, transfected with a target nucleic acid (i.e., protease or PE-like proprotein nucleic acid), and re-infused back into the subject organism (e.g., patient). Various mammalian cell types suitable for ex vivo transfection are well known to those of skill in the art (see, e.g., Freshney et al., Culture of Animal Cells, a Manual of Basic Technique, third edition Wiley-Liss, New York (1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

Detailed Description Text (151):
As indicated above, in a preferred embodiment, the packageable nucleic acid which encodes a target nucleic acid is under the control of an activated or constitutive promoter. The transfected cell(s) express functional PE-like proprotein or protease. In one particularly preferred embodiment, stem cells are used in ex vivo procedures for gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types ex vivo, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34.sup.+ cells ex vivo into clinically important immune cell types using cytokines such a GM-CSF, IFN-.gamma.and TNF-.alpha.are known (see, Inaba et al. (1992) J. Exp. Med. 176, 1693-1702, and Szabolcs et al. (1995) 154: 5851-5861).

Detailed Description Text (156):
The PE-like proprotein compositions of this invention, including PE-like proproteins proproteins and targeted PE-like proproteins (i.e., PE-like proprotein attached to a a cell specific ligand), are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of the PE molecule fusion protein dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of fusion protein in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Detailed Description Text (160):
Controlled release parenteral formulations of the PE-like protein compositions of the present invention can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems see, Banga, A. J., "Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems" Technomic Publishing Company, Inc. 1995. Lancaster, Pa., incorporated herein by reference. Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 1 .mu.m are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5 .mu.m so that only nanoparticles are administered intravenously. Microparticles are typically around

100 .mu.m in diameter and are administered subcutaneously or intramuscularly. See, e.g., Kreuter, J. 1994. "Nanoparticles," in Colloidal Drug Delivery Systems, J. Kreuter, ed., Marcel Dekker, Inc., New York, N.Y., pp. 219-342; Tice and Tabibi. 1992. "Parenteral Drug Delivery: Injectibles," in Treatise on Controlled Drug Delivery, A. Kydonieus, ed., Marcel Dekker, Inc. New York, N.Y., pp.315-339, both of of which are incorporated herein by reference. Numerous systems for controlled delivery of therapeutic proteins are known. See, e.g., U.S. Pat. Nos. 5,055,303, 5,188,837, 4,235,871, 4,501,728, 4,837,028, 4,957,735 and 5,019,369, 5,055,303; 5,514,670; 5,413,797; 5,268,164; 5,004,697; 4,902,505; 5,506,206, 5,271,961; 5,254,342 and 5,534,496, each of which is incorporated herein by reference.

Detailed Description Text (162):
PE-like proproteins are useful in the therapeutic treatment of subjects to kill cells that produce proteases that cleave a protease activatable sequence. More specifically, certain cancers can be treated in this way. This includes the treatment of prostate cancer, breast cancer or colon cancer. PE-like proproteins for treatment of prostate cancer comprise a PSA activatable sequence. A number of metastatically active cancers express urokinase. Accordingly, treatment of these cancers is generally by the use of a PE-like proprotein comprising a urokinase activatable sequence. Treatment involves administering the therapeutically effective dose of the preparation to the subject.

CLAIMS:

1. A protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hour; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

2. The PE-like proprotein of claim 1 wherein the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO:1) modified with amino acids substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280.

3. The PE-like proprotein of claim 1 wherein the protease activatable sequence is cleavable by a protease secreted by a cancer cell.

4. The PE-like proprotein of claim 1 wherein the cell recognition domain comprises an antibody that specifically binds to a cancer cell surface marker.

5. The PE-like proprotein of claim 2 wherein the protease activatable sequence is cleavable by prostate specific antigen ("PSA").

6. The PE-like proprotein of claim 2 wherein the protease activatable sequence is cleavable by urokinase.

7. The PE-like proprotein of claim 2 wherein the protease activatable sequence is cleavable by neutral endoprotease, stromelysin, collagenase, cathepsin B, or cathepsin D.

8. The PE-like proprotein of claim 2 further comprising a PE Ib domain, and wherein

said PE Ib domain, the cytotoxicity domain, and the ER retention sequence together have the sequence of domains Ib and III of native PE.

9. The PE-like proprotein of claim 3 wherein the cell recognition domain is coupled to the modified translocation domain through a peptide bond.

10. The PE-like proprotein of claim 5 wherein the protease activatable sequence is SKGSFSIQYTYHV (SEQ ID NO:11), HLGGSQQLLHNKQ (SEQ ID NO:12), or SKGKGTSSQYSNTE (SEQ ID NO:13).

11. The PE-like proprotein of claim 6 wherein the protease activatable sequence is DRVYIHPF (SEQ ID NO:3), VVCGERGFFYTP (SEQ ID NO:4), FFYTPKA (SEQ ID NO:5), KRRPVKVYP (SEQ ID NO:6), PVGKKRRPVKVY (SEQ ID NO:7), KPVGKKRRPVKV (SEQ ID NO:8), GKPVGKKRRPVK (SEQ ID NO:9), or TFAGNAVRRSVGQ (SEQ ID NO:10).

12. The PE-like proprotein of claim 8 wherein the cell recognition domain is an antibody coupled to the modified translocation domain through a peptide bond and wherein the antibody specifically binds a cancer cell surface marker.

13. A composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is substantially un-activatable by fibrin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ WD NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

16. A method for killing a cancer cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence cysteine-cysteine loop is substantially un-activatable by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

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DOCUMENT-IDENTIFIER: US 6423513 B1
TITLE: Polynucleotides encoding protease-activatable pseudomonas exotoxin a-like proproteins

Abstract Text (1):
This invention provides protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins. The proproteins comprise (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain m of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence. The invention also provides methods of using these proproteins for killing target cells.

Brief Summary Text (10):
Pseudomonas Exotoxin A ("PE") is translocated into the cytosol after a furin recognition site in domain II is cleaved by furin. Protease-activatable PE-like proproteins are engineered to replace the furin recognition site by a site recognized by a protease made or secreted by a cell targeted for death, for example, a cancer cell. Upon cleavage by the target protease, the PE-like proprotein is translocated into the cytosol where the toxin's ADP-ribosylating activity kills the cell by interfering with polypeptide elongation.

Brief Summary Text (11):
The PE-like proproteins of this invention offer several advantages. First, because they are activated by a target protease, and not by furin, their toxicity is significantly more cell-specific than native PE. Second, when the cysteine-cysteine loop of PE domain II is cleaved, the disulfide bond, before it is reduced, holds the cell-recognition domain attached to the rest of PE. Many cancer cells secrete cell-specific proteases that tend to accumulate around the cell. For example, prostate cancer cells secrete prostate specific antigen. Therefore, the proproteins of this invention may be cleaved before entering the target cell. However, the protease activatable sequence is introduced into the cysteine-cysteine loop of a domain II-like sequence of the proprotein. Therefore, the cell recognition domain is still attached upon cleavage of the proprotein outside the cell, and still is available to bind to a cell surface receptor for subsequent endocytosis. Third, by selecting a proper cell recognition domain, the toxins can be targeted to bind to specific cell types. For example, the modified PE proprotein can be administered as an immunotoxin to further increase its selective toxicity to the desired cells.

Brief Summary Text (14):
In one aspect this invention provides a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5)

an endoplasmic reticulum ("ER") retention sequence.

Brief Summary Text (15):
In one embodiment of a PE-like proprotein, the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO: 1) modified with amino acids substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280. In another embodiment the protease activatable sequence is cleavable by a protease secreted by a cancer cell. In another embodiment, the cell recognition domain comprises an antibody that specifically binds to a cancer cell surface marker. In another embodiment, the protease activatable sequence is cleavable by prostate specific antigen ("PSA"), urokinase, neutral endoprotease, stromelysin, collagenase, cathepsin B, or cathepsin D. In another embodiment, the PE Ib-like domain, the cytotoxicity domain and the ER retention sequence together have the sequence of domains Ib and III of native PE. In other embodiment, the cell recognition domain is coupled to the modified translocation domain through a peptide bond (i.e., as a fusion protein) or through a chemical linkage.

Brief Summary Text (16):
In another aspect, this invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention.

Brief Summary Text (17):
In another aspect, this invention provides a recombinant polynucleotide comprising a nucleotide sequence encoding a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the recombinant polynucleotide is an expression vector further comprising an expression control sequence operatively linked to the nucleotide sequence.

Brief Summary Text (18):
In another aspect, this invention provides a method for killing a target cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the cancer cell is, without limitation, a prostate cancer cell, a breast cancer cell or a colon cancer cell.

Brief Summary Text (19):
In another aspect, this invention provides a method for therapeutically treating a subject suffering from cancer comprising administering to the subject a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. More specifically, the PE-like proprotein comprises a protease activatable sequence that is cleavable by an enzyme produced by the cancer cell. The PE-like proprotein can be administered as a pharmaceutical composition.

Detailed Description Text (5):
As used herein, "activates" includes reference to the formation of a cytotoxic PE-like molecule having an IC50 of no more than 30 ng/ml according to cytotoxicity assays as provided, for example, in the Examples. See also, Brinkmann et al., Proc. Natl. Acad. Sci. USA, 88:8616-8620 (1991).

Detailed Description Text (48):
Protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins are polypeptides having structural domains organized, except as provided herein, in the same general sequence as the four structural domains of PE, and having certain functions (e.g., cell recognition, cytosolic translocation, cytotoxicity and endoplasmic reticulum retention) also possessed by the functional domains of PE. More specifically, the general order is: domain Ia, domain II, domain Ib, domain III. However, as described in more detail herein, domain Ia can be eliminated and

replaced by a binding protein chemically coupled to the molecule, or, a cell recognition domain can be inserted just before the ER retention sequence in domain III. Domain Ib can be eliminated. Domain II is positioned to the amino-terminal side side of domain III.

Detailed Description Text (49):
In contrast to native PE, the PE-like proproteins of this invention are engineered to eliminate the native furin cleavage site of domain II and to include within domain II of PE (and, preferentially, within a cysteine-cysteine loop of domain II) a protease activatable sequence that is cleavable by a target protease. Preferably, the target protease is a protease produced by a cell targeted for death, e.g., a cancer cell.

Detailed Description Text (50):
Accordingly, PE-like proproteins include the following structural domains comprised of amino acid sequences, the domains imparting particular functions to the proprotein: (1) a "cell recognition domain" that functions as a ligand for a cell surface receptor and that mediates binding of the protein to a cell; (2) a "translocation domain" that mediates translocation from the endosomes to the cytosol and that includes the protease-activatable sequence and is substantially unactivatable by furin; (3) an optional "PE 1b-like domain" of up to 1500 amino acids; (4) a "cytotoxic domain" that functions to kill cells, preferably, by interfering with ADP-ribosylation activity; and (5) an "endoplasmic reticulum ("ER") retention sequence" that functions to translocate the molecule from the endosome to the endoplasmic reticulum, from which it enters the cytosol.

Detailed Description Text (51):
The relationship of PE structure to its function has been extensively studied. The amino acid sequence of PE has been re-engineered to provide new functions, and many amino acids or peptide segments critical and non-critical to PE function have been identified. Accordingly, the PE-like proproteins of this invention can incorporate these structural modifications to PE within the boundaries set forth herein.

Detailed Description Text (58):
In preferred embodiments, the PE-like proproteins of the present invention can be modified with a cell specific ligand to target the PE-like proproteins to particular cells. PE-like proproteins so modified preferentially bind to cells displaying the binding partner to that ligand. In a preferred embodiment, the cell specific ligand is an antibody; thereby, the proprotein is an immunotoxin. Cell specific ligands which are proteins can often be formed in part or in whole as a fusion protein with the PE-like proproteins of the present invention. A "fusion protein" includes reference to a polypeptide formed by the joining of two or more polypeptides through a peptide bond formed by the amino terminus of one polypeptide and the carboxyl terminus of the other polypeptide. The fusion protein may be formed by the chemical coupling of the constituent polypeptides but is typically expressed as a single polypeptide from a nucleic acid sequence encoding the single contiguous fusion protein. Included among such fusion proteins are single chain Fv fragments (scFv). Particularly preferred targeted PE-like proproteins are disulfide stabilized immunotoxins which can be formed in part as a fusion protein as exemplified herein. Other protein cell specific ligands can be formed as fusion proteins using cloning methodologies well known to the skilled artisan.

Detailed Description Text (60):
In one embodiment, protease-activatable PE-like proproteins are immunotoxins directed against particular cancer cells. Accordingly, the cell recognition domain is an antibody that specifically binds to a cell surface receptor or marker specific for a cancer cell. This includes, for example, prostate cancer cells, breast cancer cells or colon cancer cells. In alternative embodiments, the proprotein can be a fusion protein or a conjugate protein. In the fusion protein, domain Ia is replaced with a polypeptide sequence for an immunoglobulin heavy chain

from an immunoglobulin specific for the cancer cell. The light chain of the immunoglobulin can be co-expressed with the proprotein so as to form a light chain-heavy chain dimer. In the conjugate protein, the antibody is chemically linked to a polypeptide comprising the other domains of the proprotein.

Detailed Description Text (61):
The procedure for attaching a PE-like proprotein to an antibody or other cell specific ligand will vary according to the chemical structure of the toxin. Antibodies contain a variety of functional groups; e.g., sulfhydryl (--S), carboxylic acid (COOH) or free amine (--NH.sub.2) groups, which are available for reaction with a suitable functional group on a toxin. Additionally, or alternatively, the antibody or PE-like proprotein can be derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Ill.

Detailed Description Text (62):
A bifunctional linker having one functional group reactive with a group on the PE-like proprotein, and another group reactive with a cell specific ligand, can be used used to form a desired conjugate. Alternatively, derivatization may involve chemical treatment of the PE-like proprotein or the cell specific ligand, e.g., glycol cleavage of the sugar moiety of a glycoprotein antibody with periodate to generate free aldehyde groups. The free aldehyde groups on the antibody may be reacted with free amine or hydrazine groups on the antibody to bind the PE-like proprotein thereto. (See U.S. Pat. No. 4,671,958). Procedures for generation of free sulfhydryl groups on antibodies or other proteins, are also known (See U.S. Pat. No. 4,659,839).

Detailed Description Text (64):
In some circumstances, it is desirable to free the PE-like proprotein from the antibody or other cell specific ligand when the conjugate has reached its target site. Therefore, conjugates comprising linkages which are cleavable in the vicinity or within the target site may be used when the toxin is to be released at the target site. Cleaving of the linkage to release the agent from the ligand may be prompted by enzymatic activity or conditions to which the immunoconjugate is subjected either inside the target cell or in the vicinity of the target site. A number of different cleavable linkers are known to those of skill in the art. See U.S. Pat. Nos. 4,618,492; 4,542,225, and 4,625,014. SPDP is a reversible NHS-ester, pyridyl disulfide cross-linker used to conjugate amine-containing molecules to sulfhydryls. Another chemical modification reagent is 2-iminothiolane which reacts with amines and yields a sulfhydryl. Water soluble SPDP analogs, such as Sulfo-LC-SPDP (Pierce, Rockford, Ill.) are also available. SMPT is a reversible NHS-ester, pyridyl disulfide cross-linker developed to avoid cleavage in vivo prior to reaching the antigenic target. Additionally, the NHS-ester of SMPT is relatively stable in aqueous solutions.

Detailed Description Text (65):
C. PE-Like Translocation Domain

Detailed Description Text (66):
Protease-activatable PE-like proproteins also comprise an amino acid sequence encoding a "modified PE translocation domain." The modified PE translocation domain includes a cysteine-cysteine loop that includes a protease activatable sequence, and is engineered to eliminate the native furin recognition site. It comprises an amino acid sequence sufficient to effect translocation of chimeric proteins that have been endocytosed by the cell into the cytosol. A proprotein that does not contain the sequence RXXR (SEQ. ID NO:30) within the cysteine-cysteine loop is substantially un-activatable by furin and, consequently, substantially non-toxic to cells not having an enzyme that cleaves the protease-activatable sequence. The amino acid sequence generally is substantially identical to a sequence selected

from domain II of PE. Upon cleavage by a protease that recognizes the protease-activatable sequence and reduction of the disulfide bond, the proprotein is now activated, i.e., is enabled for translocation into the cytosol and subsequent cytotoxic activity.

Detailed Description Text (67):
More specifically, protease-activatable PE-like proproteins comprise a cysteine-cysteine loop that comprises the protease activatable sequence. The loop generally has between about 10 and 50 amino acids, more preferably, about 23 amino acids, as in native PE. The protease activatable sequence can be located anywhere within the cysteine-cysteine loop. Preferably, the sequence is more than 5 amino acids from the cysteine residues involved in the disulfide bond. More preferably, the cysteine-cysteine loop has the sequence of the loop in native PE except for substitutions that introduce the protease activatable sequence. The cysteine-cysteine loop also is modified to eliminate the native furin recognition sequence. Furin recognizes the sequence RXXR (SEQ ID NO:30), wherein X is any amino acid. This sequence occurs in native PE at amino acids 276-279.

Detailed Description Text (73):
Amino acid sequences that can serve as protease activatable sequences are chosen from peptide substrates of the desired protease. Proteases and their amino acid sequence substrates are well known in the art. For example, urokinase is an enzyme found in many metastatically active cancers. The sequence of urokinase is described by Nagai et al., Gene, 36:183-188 (1985); Riccio et al. Nucl. Acids Res., 13:2759-2771 (1985); and, Holmes et al., Bio/Technology 3:923-929 (1985), all of which are incorporated herein by reference. Peptide substrates for urokinase ("urokinase activatable sequences) include the sequences: DR/VYIHPF (SEQ ID NO:3) from angiotensin, VVCGER/GFFYTP (SEQ ID NO:4) from the insulin B chain, FFYTPK/A (SEQ ID NO:5), and from adrenal corticotrophic hormone (ACTH) the sequences: KRRPVK/VYP (SEQ ID NO:6), PVGKKR/RPVKVY (SEQ ID NO:7), KPVGKK/RRPVKV (SEQ ID NO:8), and GKPVGK/KRRPVK (SEQ ID NO:9), where "/" indicates the cleavage site. In particularly preferred embodiments, the urokinase activatable sequence has the sequence TFAGNAVRR/SVGQ (SEQ ID NO:10). Generally, SEQ ID NO: 10 is inserted between residues 271 and 283 of the PE-like proprotein. The sequences of the urokinase activatable sequences disclosed above can be shortened by one or two amino acid residues at the carboxyl and/or amino terminal ends such that at least two residues of the prototype sequences are maintained on either side of the cleavage site.

Detailed Description Text (74):
In other embodiments, the protease activatable sequence is derived from any substrate of prostate-specific antigen ("PSA"). This includes, for example, semenogelin I or semenogelin II or insulin-like growth factor binding protein. A preferred protease activatable sequence is derived from semenogelin I. Protease activatable sequences from semenogelin I (PSA activatable sequences) include: sem 1 having the sequence SKGSFSIQY/TYHV (SEQ ID NO:11), sem 3 having the sequence HLGGSQQLL/HNKQ (SEQ ID NO:12), and sem 5 having the sequence SKGKGTSSQY/SNTE (SEQ ID NO:13), where "/" indicates the cleavage at position 279. In particularly preferred embodiments the sem 1, sem 3, and sem 5 protease activatable sequences are substituted for the native PE-like proprotein amino acids residing between residues 271 and 283. The sequences of the PSA activatable sequences disclosed above can be shortened by one or two amino acid residues at the carboxyl and/or amino terminal ends such that at least two residues of the prototype sequences are maintained on either side of the cleavage site.

Detailed Description Text (79):
Protease activatable PE-like proproteins optionally include an amino acid sequence encoding a "PE 1b-like domain." The PE 1b-like domain is located at the native Ib domain location of PE, between the translocation domain (e.g., domain II) and the cytotoxic domain (e.g., domain III). The PE 1b-like domain can be up to about 1500 amino acids. This includes domains having between about 15 amino acids and about

350 amino acids or about 15 amino acids and about 50 amino acids.

Detailed Description Text (82):
PE-like proproteins also comprise an amino acid sequence encoding a "cytotoxicity domain" and an "endoplasmic reticulum (`ER`) retention sequence." The cytotoxicity domain has a sequence sufficiently complementary to domain III of native PE to confer ADP-ribosylating activity, thereby rendering the construct cytotoxic. The ER retention sequence functions in translocating the proprotein to from the endosome to the endoplasmic reticulum, from where it is transported to the cytosol. The cytotoxic domain is located at the position of domain III in PE.

Detailed Description Text (88):
F. Methods Of Making PE-like Proproteins

Detailed Description Text (89):
PE-like proproteins preferably are produced recombinantly, as described below. This invention also envisions the production of PE chimeric proteins by chemical synthesis using methods available to the art.

Detailed Description Text (90):
G. Testing PE-like Proproteins

Detailed Description Text (91):
Having selected various structures as domains of the proprotein, the function of these domains, and of the proprotein as a whole, can be tested to detect functionality. PE-like proproteins can be tested for cell recognition, cleavability, cleavability, cytosolic translocation and cytotoxicity using routine assays. The entire proprotein protein can be tested, or, the function of various domains can be tested by substituting them for native domains of the wild-type toxin.

Detailed Description Text (104):
In another method, the ability of the translocation domain and ER retention sequence to effect translocation to the cytosol can be tested with a construct containing a domain III having ADP ribosylation activity. Briefly, cells are seeded in tissue culture plates and exposed to the PE-like proprotein or to an engineered PE exotoxin containing the modified translocation domain or ER retention sequence in place of the native domains. ADP ribosylation activity is determined as a function of inhibition of protein synthesis by, e.g., monitoring the incorporation of .sup.3 H-leucine.

Detailed Description Text (105):
III. RECOMBINANT POLYNUCLEOTIDES ENCODING PE-LIKE PROPROTEINS

Detailed Description Text (108):
With the protease activatable sequences herein provided, and the PE sequence as disclosed in SEQ ID NO:1, one of skill can readily construct a variety of clones containing functionally equivalent nucleic acids, such as nucleic acids which differ in sequence but which encode the same PE-like proprotein. PE-like proprotein related compositions such as PE-like proprotein fusion proteins comprising a cell-specific ligand can also be constructed. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art and exemplified herein. Other examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or Current Protocols in Molecular Biology, (Ausubel, et al. (eds.), Greene Publishing and Wiley-Interscience, New York (1987).

Detailed Description Text (110):
Polynucleotides encoding PE-like proproteins or subsequences thereof, such as a protease activatable sequence, can be prepared by any suitable method including, for example, cloning and restriction of appropriate sequences as discussed supra, or by direct chemical synthesis by methods such as the phosphotriester method of Narang et al. Meth. Enzymol. 68: 90-99 (1979); the phosphodiester method of Brown et al., Meth. Enzymol. 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage et al., Tetra. Lett., 22: 1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Letts., 22(20):1859-1862, e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168; and, the solid support method of U.S. Pat. No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Detailed Description Text (111):
Nucleic acids encoding native PE exotoxin can be modified to form the PE-like proproteins of the present invention. Modification by site-directed mutagenesis is well known in the art. Native PE exotoxin nucleic acids can be amplified by in vitro methods. Amplification methods include the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (SSR). A wide variety of cloning methods, host cells, and in vitro amplification methodologies are well-known to persons of skill.

Detailed Description Text (113):
Once the nucleic acids encoding an PE-like proprotein of the present invention is isolated and cloned, one may express the desired protein in a recombinantly engineered cell such as bacteria, yeast, insect and mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of proteins. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made. In brief, the expression of natural or synthetic nucleic acids encoding the isolated proteins of the invention will typically be achieved by operably linking the DNA or cDNA to an expression control sequence (e.g., a promoter which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding the protein. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator.

Detailed Description Text (114):
One of skill would recognize that modifications can be made to a nucleic acid encoding a PE-like proprotein without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

Detailed Description Text (117):
Nucleic acids encoding PE-like proproteins of the present invention may be expressed expressed in a variety of host cells, including E. coli, other bacterial hosts,

yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines lines and myeloma cell lines. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For E. coli this includes includes a promoter such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, etc., and a polyadenylation sequence, and may include splice donor and acceptor sequences. The plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for E. coliand calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

Detailed Description Text (118):
Once expressed, the recombinant fusion proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, R. Scopes, Protein Purification, Springer-Verlag, N.Y. (1982)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the PE-like proproteins of the present invention may then be used therapeutically.

Detailed Description Text (119):
The PE-like proproteins of the present invention can also be constructed in whole or or in part using standard synthetic methods. Solid phase synthesis of isolated proteins of the present invention of less than about 50 amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany and Merrifield, Solid-Phase Peptide Synthesis; pp. 3-284 in The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A., Merrifield, et al. J. Am. Chem. Soc., 85: 2149-2156 (1963), and Stewart et al., Solid Phase Peptide Synthesis, 2nd ed. Pierce Chem. Co., Rockford, Ill. (1984). Proteins of greater length may be synthesized by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N, N'-dicycylohexylcarbodiimide)) is known to those of skill.

Detailed Description Text (120):
Eliminating nucleotides encoding amino acids 1-252 yields a construct referred to as "PE40." Eliminating nucleotides encoding amino acids 1-279 yields a construct referred to as "PE37." (See Pastan et al., U.S. Pat. No. 5,602,095.) The practitioner can ligate sequences encoding cell recognition domains to the 5' end of these platforms to engineer PE-like chimeric proteins that are directed to particular cell surface receptors. These constructs optionally can encode an amino-terminal methionine. A cell recognition domain can be inserted into such constructs in the nucleotide sequence encoding the ER retention sequence.

Detailed Description Text (123):
In preferred embodiment, the PE-like proproteins of the present invention are attached to antibodies to form immunotoxins. Particularly preferred are disulfide stabilized antibodies as exemplified herein. The attachment may be by covalent or non-covalent means (e.g., biotin and avidin). Typically, covalent attachment can be accomplished by construction of fusion proteins or by the use of chemical linkers as discussed, supra. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

Detailed Description Text (136):
In another embodiment, this invention provides for fully human antibodies which serve as cell specific ligands for construction of PE-like proproteins. Human antibodies consist entirely of characteristically human polypeptide sequences. The human antibodies of this invention can be produced in using a wide variety of methods (see, e.g., Larrick et al., U.S. Pat. No. 5,001,065, for review).

Detailed Description Text (140):
The present invention provides nucleic acids encoding proteases for cleavage of the desired protease activatable sequence (protease nucleic acids). The mammalian cells can be altered to express the cognate protease to a particular protease activatable sequence. Thus, mammalian cells can be altered for susceptibility to a particular PE-like proprotein.

Detailed Description Text (141):
The present invention also provides nucleic acids encoding the PE-like proprotein compositions of the present invention (PE-like proprotein nucleic acids). These PE-like proproteins can be used to inhibit protein synthesis in recombinant or native cells expressing the cognate protease.

Detailed Description Text (147):
Ex vivo mammalian cell transfection for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject mammalian organism, transfected with a target nucleic acid (i.e., protesae or PE-like proprotein nucleic acid), and re-infused back into the subject organism (e.g., patient). Various mammalian cell types suitable for ex vivo transfection are well known to those of skill in the art (see, e.g., Freshney et al., Culture of Animal Cells, a Manual of Basic Technique, third edition Wiley-Liss, New York (1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

Detailed Description Text (148):
As indicated above, in a preferred embodiment, the packageable nucleic acid which encodes a target nucleic acid is under the control of an activated or constitutive promoter. The transfected cell(s) express functional PE-like proprotein or protease. In one particularly preferred embodiment, stem cells are used in ex vivo procedures for gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types ex vivo, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34.sup.+ cells ex vivo into clinically important immune cell types using cytokines such a GM-CSF, IFN-.gamma. and TNF-.alpha. are known (see, Inaba et al. (1992) J. Exp. Med. 176, 1693-1702, and Szabolcs et al. (1995) 154: 5851-5861).

Detailed Description Text (153):
The PE-like proprotein compositions of this invention, including PE-like proproteins proproteins and targeted PE-like proproteins (i.e., PE-like proprotein attached to a a cell specific ligand), are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of the PE molecule fusion protein dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of fusion protein in these

formulations can vary widely, and will be selected primarily based on fluid volumes, volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Detailed Description Text (157):
Controlled release parenteral formulations of the PE-like protein compositions of the present invention can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems see, Banga, A. J., "Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems" Technomic Publishing Company, Inc. 1995. Lancaster, Pa., incorporated herein by reference. Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 1 .mu.m are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5 .mu.m so that only nanoparticles are administered intravenously. Microparticles are typically around 100 .mu.m in diameter and are administered subcutaneously or intramuscularly. See, e.g., Kreuter, J. 1994. "Nanoparticles," in Colloidal Drug Delivery Systems, J. Kreuter, ed., Marcel Dekker, Inc., New York, N.Y., pp. 219-342; Tice and Tabibi. 1992. "Parenteral Drug Delivery: Injectibles," in Treatise on Controlled Drug Delivery, A. Kydonieus, ed., Marcel Dekker, Inc. New York, N.Y., pp.315-339, both of which are incorporated herein by reference. Numerous systems for controlled delivery of therapeutic proteins are known. See, e.g., U.S. Pat. Nos. 5,055,303, 5,188,837, 4,235,871, 4,501,728, 4,837,028 4,957,735 and 5,019,369, 5,055,303; 5,514,670; 5,413,797; 5,268,164; 5,004,697; 4,902,505; 5,506,206, 5,271,961; 5,254,342 and 5,534,496, each of which is incorporated herein by reference.

Detailed Description Text (159):
PE-like proproteins are useful in the therapeutic treatment of subjects to kill cells that produce proteases that cleave a protease activatable sequence. More specifically, certain cancers can be treated in this way. This includes the treatment of prostate cancer, breast cancer or colon cancer. PE-like proproteins for treatment of prostate cancer comprise a PSA activatable sequence. A number of metastatically active cancers express urokinase. Accordingly, treatment of these cancers is generally by the use of a PE-like proprotein comprising a urokinase activatable sequence. Treatment involves administering the therapeutically effective dose of the preparation to the subject.

CLAIMS:

1. A recombinant polynucleotide comprising a nucleotide sequence encoding a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain of between 10 and 1500 amino acids that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

2. The recombinant polynucleotide of claim 1, further comprising a nucleic acid sequence encoding a PE Ib-like domain comprising an amino acid sequence of between 5 and about 1500 amino acids, which amino acid sequence is positioned between the modified PE translocation domain and the cytotoxicity domain and which does not

interfere with the ability of the PE-like proprotein to bind cells, translocate, or ribosylate ADP.

☐ 4. Document ID: US 5460739 A

L13: Entry 4 of 34 File: USPT Oct 24, 1995

DOCUMENT-IDENTIFIER: US 5460739 A
TITLE: Star polymer viscosity index improver for oil compositions

Detailed Description Text (6):
The star polymer was next hydrogenated at 75.degree. C. to saturate greater than 98% the olefinic unsaturation originally contained in the isoprene and butadiene blocks using a catalyst prepared by combining nickel ethylhexanoate and triethylaluminum (2.3 moles of aluminum per mole of nickel). The hydrogenation catalyst was then extracted by an aqueous acidic water-wash at room temperature, using a high dilution ratio. Steam/hot water devolatilization was used to coagulate the polymer. Differential scanning calorimetry was used to determine the melting point of the PE-like blocks. The melting point occurred at 89.degree. C. The data and results are summarized in Table 1.

Detailed Description Text (12):
In this example, a star polymer comprising hydrogenated poly(isoprene-butadiene) diblock copolymer arms was prepared using the procedure summarized in Example 1 except that the number average molecular weight of the polyisoprene blocks by gel permeation chromatography were 31,700 and the polybutadiene blocks were 15,500. Nmr analysis indicated that 90.8 percent of the butadiene had been incorporated by 1,4-addition, and the total butadiene represented 29.9 percent of the total polydiene content of the arms. After coupling with divinylbenzene, analysis of the coupled, unhydrogenated star polymer indicated that the 1,4-addition of butadiene was 90.9 percent, and that the butadiene contained therein was 29.9 percent. The star polymer was next hydrogenated to remove greater than 99% of the olefinic unsaturation originally contained in the isoprene and butadiene blocks. The polymer solution became extremely viscous upon hydrogenation; it was diluted further with cyclohexane before acid-wash and polymer separation. The melting point of the PE-like blocks was determined to be 98.5.degree. C. by differential scanning calorimetry. The data and results are summarized in Table 1.

Detailed Description Text (15):
In this example, a star polymer comprising hydrogenated poly(isoprene-butadiene) diblock copolymer arms was prepared using the procedure summarized in Example 1 except that the molecular weight of the polyisoprene blocks by gel permeation chromatography were 58,000 and the polybutadiene blocks were 11,200. Nmr analysis indicated that 90.6 percent of the butadiene had been incorporated by 1,4-addition, and the butadiene represented 14.7 percent of the total polydiene content of the arms. After coupling with divinylbenzene, analysis of the coupled, unhydrogenated star polymer indicated that the 1,4-addition of butadiene was 90.2 percent, and that butadiene represented 14.5 percent of the polydiene content. The star polymer was next hydrogenated so as to saturate greater than 98% of the olefinic unsaturation originally contained in the isoprene and butadiene blocks. The melting point of the PE-like blocks was determined to be 95.0.degree. C. by differential

scanning calorimetry. The data and results are summarized in Table 1.

Detailed Description Text (21):
In this example, a star polymer comprising hydrogenated poly(isoprene-butadiene) diblock copolymer arms was prepared using the procedure summarized in Example 1 except that a ratio of 4 moles of divinylbenzene per mole of living polymer was used for coupling purposes and the molecular weight of the polyisoprene blocks by gel permeation chromatography were 38,800 and the polybutadiene blocks were 10,400. It was estimated that the butadiene represented 21.2 percent of the total polydiene content of the arms. The star polymer was hydrogenated and the resultant solution was aqueous acid and water washed at 90.degree. C. Nmr analysis of the final product showed that it was 99.2 percent of the olefinic unsaturation had been removed by hydrogenation. The melting point of the PE-like blocks was determined to be 94.9.degree. C. by differential scanning calorimetry. The data and results are summarized in Table 1.

Detailed Description Text (26):
In this example, a star polymer comprising hydrogenated poly(isoprene-butadiene-isoprene) triblock copolymer arms was prepared using the procedure summarized in Example 1 except that after the poly(isoprene-butadiene) blocks were incorporated, isoprene monomer was again added to the living polymer. The molecular weight of the outer polyisoprene blocks by gel permeation chromatography were 34,200, the polybutadiene blocks were 5,300, and the inner polyisoprene blocks were 11,100. NMR analysis of the triblock polymer arms indicated that 10.5 percent of butadiene was incorporated into the block copolymer and that 90.0 percent of the butadiene polymerized by 1,4-addition. After coupling with divinylbenzene, the star polymer cement was reduce from 20 percent to 10 percent solids content, hydrogenated at 65.degree. C. and the resultant reaction mixture aqueous acid and water washed at 50.degree. C. It was subsequently determine that 99.3 percent of the olefinic unsaturation originally contained in the isoprene and butadiene blocks had been removed by hydrogenation. The melting point of the PE-like blocks was determined to be 84.8.degree. C. by differential scanning calorimetry. The data and results are summarized in Table 1.

☐ 5. Document ID: US 5193899 A

L13: Entry 5 of 34 File: USPT Mar 16, 1993

DOCUMENT-IDENTIFIER: US 5193899 A
TITLE: Planar light-source device and illumination apparatus using the same

Detailed Description Paragraph Table (3):
TABLE 3 _____ DISTANCE OF d.sub.2 1 2 4 8 16 23 (mm) WIDTH OF PE-LIKE 3 7 13 20 35 45 DARK PORTION (mm)

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 6. Document ID: US 5089944 A

L13: Entry 6 of 34 File: USPT Feb 18, 1992

DOCUMENT-IDENTIFIER: US 5089944 A
TITLE: Planar light-source device and illumination apparatus using the same

Detailed Description Paragraph Table (3):
TABLE 3 _____ DISTANCE OF d.sub.2 1 2 4 8 16 23 (mm) WIDTH OF PE-LIKE 3 7 13 20 35 45 DARK PORTION (mm)

_____

☐ 7. Document ID: US 5034864 A

L13: Entry 7 of 34 File: USPT Jul 23, 1991

DOCUMENT-IDENTIFIER: US 5034864 A
TITLE: Planar light-source device and illumination apparatus using the same

Detailed Description Paragraph Table (3):
TABLE 3 _____ DISTANCE OF d.sub.2 1 2 4 8 16 23 (mm) WIDTH OF PE-LIKE 3 7 13 20 35 45 DARK PORTION (mm)

_____

☐ 8. Document ID: WO 9820135 A2

L13: Entry 8 of 34 File: EPAB May 14, 1998

PUB-NO: WO009820135A2
DOCUMENT-IDENTIFIER: WO 9820135 A2
TITLE: PROTEASE-ACTIVATABLE PSEUDOMONAS EXOTOXIN A-LIKE PROPROTEINS

PUBN-DATE: May 14, 1998

INVENTOR-INFORMATION:

NAME	COUNTRY
FITZGERALD, DAVID J	US
REITER, YORAM	IL
PASTAN, IRA	US

INT-CL (IPC): C12 N 15/31; C07 K 14/21; C07 K 19/00; C12 N 15/62; C07 K 16/30; A61 K 39/104; A61 K 47/48
EUR-CL (EPC): A61K047/48; C07K014/21, C07K016/30

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc

☐ 9. Document ID: WO 9902713 A1, AU 9884813 A, EP 1000163 A1, AU 731703 B, JP 2001510683 W

L13: Entry 9 of 34 File: DWPI Jan 21, 1999

DERWENT-ACC-NO: 1999-120914
DERWENT-WEEK: 199910

TITLE: New Pseudomonas exotoxin chimeric immunogens - comprise a non-native epitope for producing an immune response to pathogens, e.g. virus, bacteria, or protozoa or to cancer antigens

INVENTOR: FITZGERALD, D J

PRIORITY-DATA: 1997US-052375P (July 11, 1997)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
WO 9902713 A1	January 21, 1999	E	091	C12N015/62
AU 9884813 A	February 8, 1999		000	C12N015/62
EP 1000163 A1	May 17, 2000	E	000	C12N015/62
AU 731703 B	April 5, 2001		000	C12N015/62
JP 2001510683 W	August 7, 2001		109	C12N015/09

INT-CL (IPC): A61 K 39/002; A61 K 39/02; A61 K 39/104; A61 K 39/12; A61 K 39/21; A61 K 48/00; A61 P 31/00; A61 P 31/18; A61 P 37/04; A61 P 43/00; C07 K 16/10; C12 N 15/09; C12 N 15/62; C12 N 15/70

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc

☐ 10. Document ID: US 20030054012 A1, WO 9902712 A1, AU 9883929 A, EP 1000162 A1

L13: Entry 10 of 34 File: DWPI Mar 20, 2003

DERWENT-ACC-NO: 1999-120913
DERWENT-WEEK: 200323

TITLE: New Pseudomonas exotoxin chimeric immunogens - comprise a foreign epitope for producing an immune response to pathogens, e.g. virus, bacteria or protozoa or to cancer antigens

INVENTOR: FITZGERALD, D J; MRSNY, R J

PRIORITY-DATA: 1997US-056924P (July 11, 1997), 2000US-0462713 (May 12, 2000)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 20030054012 A1	March 20, 2003		000	A61K039/02

WO 9902712 A1	January 21, 1999	E	085	C12N015/62
AU 9883929 A	February 8, 1999		000	C12N015/62
EP 1000162 A1	May 17, 2000	E	000	C12N015/62

INT-CL (IPC): A61 K 39/02; A61 K 39/104; A61 K 39/21; C07 K 16/10; C12 N 15/62; C12 Q 1/70

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 11. Document ID: WO 9820135 A2, AU 9852474 A, EP 941334 A2, AU 716564 B, JP 2001504334 W, US 6423513 B1, US 6426075 B1

L13: Entry 11 of 34 File: DWPI May 14, 1998

DERWENT-ACC-NO: 1998-286951
DERWENT-WEEK: 200271
COPYRIGHT 2004 DERWENT INFORMATION LTD
TITLE: Pseudomonas exotoxin A-like proprotein which is protease-activatable - allows activation by desired protease through protease activatable sequence in domain II loop, useful to selectively kill e.g. cancer cells

INVENTOR: FITZGERALD, D J; PASTAN, I ; REITER, Y

PRIORITY-DATA: 1996US-030376P (November 6, 1996), 1999US-0297851 (July 30, 1999), 2000US-0479479 (January 10, 2000)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
WO 9820135 A2	May 14, 1998	E	075	C12N015/31
AU 9852474 A	May 29, 1998		000	C12N015/31
EP 941334 A2	September 15, 1999	E	000	C12N015/31
AU 716564 B	March 2, 2000		000	C12N015/31
JP 2001504334 W	April 3, 2001		092	C12N015/09
US 6423513 B1	July 23, 2002		000	C12P021/04
US 6426075 B1	July 30, 2002		000	A61K039/108

INT-CL (IPC): A61 K 38/00; A61 K 39/00; A61 K 39/104; A61 K 39/108; A61 K 47/48; A61 P 35/00; C07 K 14/21; C07 K 16/30; C07 K 19/00; C12 N 15/09; C12 N 15/31; C12 N 15/62; C12 P 21/04; C12 P 21/06

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 12. Document ID: US 3473629 A

L13: Entry 12 of 34 File: USOC Oct 21, 1969

US-PAT-NO: 3473629
DOCUMENT-IDENTIFIER: US 3473629 A

TITLE: ENGINE OIL RECONDITIONER

DATE-ISSUED: October 21, 1969

INVENTOR-NAME: ROBINSON LUTHER; ROLAND EDGAR G

US-CL-CURRENT: 184/6.22, 208/179

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw D

☐ 13. Document ID: US 3445393 A

L13: Entry 13 of 34 File: USOC May 20, 1969

US-PAT-NO: 3445393
DOCUMENT-IDENTIFIER: US 3445393 A

TITLE: PACKING AND SEALING COMPOSITION

DATE-ISSUED: May 20, 1969

INVENTOR-NAME: HINDS CYRIL

US-CL-CURRENT: 508/181; 184/109, 184/37, 264/242, 277/540, 277/944, 508/106, 508/590

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw D

☐ 14. Document ID: US 3395896 A

L13: Entry 14 of 34 File: USOC Aug 6, 1968

US-PAT-NO: 3395896
DOCUMENT-IDENTIFIER: US 3395896 A

TITLE: Apparatus for treating soil

DATE-ISSUED: August 6, 1968

INVENTOR-NAME: FUNK WILLIAM E; LEHMAN IRVIN H

US-CL-CURRENT: 366/316

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw D

☐ 15. Document ID: US 3260412 A

L13: Entry 15 of 34 File: USOC Jul 12, 1966

US-PAT-NO: 3260412
DOCUMENT-IDENTIFIER: US 3260412 A

TITLE: Dispensing container with collapse securing means

DATE-ISSUED: July 12, 1966

INVENTOR-NAME: LARKIN MARK E

US-CL-CURRENT: 222/107; 138/119, 138/121, D09/302

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw De

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☐ 16. Document ID: US 3247744 A

L13: Entry 16 of 34 File: USOC Apr 26, 1966

US-PAT-NO: 3247744
DOCUMENT-IDENTIFIER: US 3247744 A

TITLE: Apparatus for cross cutting traveling strip materials

DATE-ISSUED: April 26, 1966

INVENTOR-NAME: HUCK WILLIAM F; SANDOR GEORGE N

US-CL-CURRENT: 83/107, 83/323, 83/325, 83/337, 83/346

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw De

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☐ 17. Document ID: US 3162000 A

L13: Entry 17 of 34 File: USOC Dec 22, 1964

US-PAT-NO: 3162000
DOCUMENT-IDENTIFIER: US 3162000 A

TITLE: Method of sealing two-piece gelatin capsules

DATE-ISSUED: December 22, 1964

INVENTOR-NAME: MAX KRAVEN

US-CL-CURRENT: 53/471; 53/900

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw De

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☐ 18. Document ID: US 3128513 A

L13: Entry 18 of 34 File: USOC Apr 14, 1964

US-PAT-NO: 3128513
DOCUMENT-IDENTIFIER: US 3128513 A

TITLE: Moldless metal casting process

DATE-ISSUED: April 14, 1964

INVENTOR-NAME: CHARLTON JOSEPH W; COTSWORTH JOHN L

US-CL-CURRENT: 164/486, 164/424, 164/441, 164/444

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 19. Document ID: US 3034474 A

L13: Entry 19 of 34 File: USOC May 15, 1962

US-PAT-NO: 3034474
DOCUMENT-IDENTIFIER: US 3034474 A

TITLE: Control board panel

DATE-ISSUED: May 15, 1962

INVENTOR-NAME: WASSELL GEORGE W

US-CL-CURRENT: 116/325; 40/446

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 20. Document ID: US 2950521 A

L13: Entry 20 of 34 File: USOC Aug 30, 1960

US-PAT-NO: 2950521
DOCUMENT-IDENTIFIER: US 2950521 A

TITLE: Permanent crimping process

DATE-ISSUED: August 30, 1960

INVENTOR-NAME: WHEAT VERNON D; HADFIELD SR WALTER

US-CL-CURRENT: 28/155, 28/166, 28/167, 28/218, 28/279

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 21. Document ID: US 2842972 A

L13: Entry 21 of 34 File: USOC Jul 15, 1958

US-PAT-NO: 2842972
DOCUMENT-IDENTIFIER: US 2842972 A

TITLE: Vehicles with expanding bodies

DATE-ISSUED: July 15, 1958

INVENTOR-NAME: ALBERT HOUDART DOMINIQUE PAUL

US-CL-CURRENT: 74/422; 254/97, 296/175, 296/26.13, 409/332

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 22. Document ID: US 2764793 A

L13: Entry 22 of 34 File: USOC Oct 2, 1956

US-PAT-NO: 2764793
DOCUMENT-IDENTIFIER: US 2764793 A

TITLE: Slider for sliding clasp fasteners

DATE-ISSUED: October 2, 1956

INVENTOR-NAME: ERNST SANDER

US-CL-CURRENT: 24/428; 24/400

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 23. Document ID: US 2646877 A

L13: Entry 23 of 34 File: USOC Jul 28, 1953

US-PAT-NO: 2646877
DOCUMENT-IDENTIFIER: US 2646877 A

TITLE: Package for tapelike material

DATE-ISSUED: July 28, 1953

INVENTOR-NAME: SCHOLL WILLIAM M

US-CL-CURRENT: 206/409

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 24. Document ID: US 2387503 A

L13: Entry 24 of 34 File: USOC Oct 23, 1945

US-PAT-NO: 2387503
DOCUMENT-IDENTIFIER: US 2387503 A

TITLE: Display device

DATE-ISSUED: October 23, 1945

INVENTOR-NAME: ELLIS EUGENE D

US-CL-CURRENT: 47/41.01; 312/117, 47/41.12

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw. De

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☐ 25. Document ID: US 2260054 A

L13: Entry 25 of 34 File: USOC Oct 21, 1941

US-PAT-NO: 2260054
DOCUMENT-IDENTIFIER: US 2260054 A

TITLE: Turn spacing device for coils

DATE-ISSUED: October 21, 1941

INVENTOR-NAME: PLATT STEPHEN A

US-CL-CURRENT: 140/89; 264/DIG.40

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw. De

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☐ 26. Document ID: US 1977319 A

L13: Entry 26 of 34 File: USOC Oct 16, 1934

US-PAT-NO: 1977319
DOCUMENT-IDENTIFIER: US 1977319 A

TITLE: Drain flushing device

DATE-ISSUED: October 16, 1934

INVENTOR-NAME: MCEWAN JAMES J

US-CL-CURRENT: 4/255.06; 134/168C, 4/255.01

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw. De

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☐ 27. Document ID: US 1950219 A

L13: Entry 27 of 34 File: USOC Mar 6, 1934

US-PAT-NO: 1950219
DOCUMENT-IDENTIFIER: US 1950219 A

TITLE: Spun glass method and apparatus

DATE-ISSUED: March 6, 1934

INVENTOR-NAME: BLUM HANS J

US-CL-CURRENT: 65/479; 65/535

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 28. Document ID: US 1947465 A

L13: Entry 28 of 34 File: USOC Feb 20, 1934

US-PAT-NO: 1947465
DOCUMENT-IDENTIFIER: US 1947465 A

TITLE: Textile fabric

DATE-ISSUED: February 20, 1934

INVENTOR-NAME: HENRY DREYFUS

US-CL-CURRENT: 428/152; 242/173, 442/208, 57/251

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 29. Document ID: US 1897861 A

L13: Entry 29 of 34 File: USOC Feb 14, 1933

US-PAT-NO: 1897861
DOCUMENT-IDENTIFIER: US 1897861 A

TITLE: Casing for key-bows

DATE-ISSUED: February 14, 1933

INVENTOR-NAME: PRYDE QUAY MARION

US-CL-CURRENT: 40/330; 428/913

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 30. Document ID: US 1828004 A

L13: Entry 30 of 34 File: USOC Oct 20, 1931

US-PAT-NO: 1828004
DOCUMENT-IDENTIFIER: US 1828004 A

TITLE: Electrolytic cell curtain

DATE-ISSUED: October 20, 1931

INVENTOR-NAME: WARD LOUIS E

US-CL-CURRENT: 204/247; 204/279

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWOC | Draw De

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☐ 31. Document ID: US 1822223 A

L13: Entry 31 of 34 File: USOC Sep 8, 1931

US-PAT-NO: 1822223
DOCUMENT-IDENTIFIER: US 1822223 A

TITLE: Construction of ships, boats and the like

DATE-ISSUED: September 8, 1931

INVENTOR-NAME: RUDOLF KLINGER JOHANN

US-CL-CURRENT: 114/67A

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWOC | Draw De

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☐ 32. Document ID: US 1614682 A

L13: Entry 32 of 34 File: USOC Jan 18, 1927

US-PAT-NO: 1614682
DOCUMENT-IDENTIFIER: US 1614682 A

TITLE: OCR SCANNED DOCUMENT

DATE-ISSUED: January 18, 1927

INVENTOR-NAME: Name not available

US-CL-CURRENT: 177/173; 177/180, 177/187, 177/219, 177/221

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWOC | Draw De

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☐ 33. Document ID: US 1533658 A

L13: Entry 33 of 34 File: USOC Apr 14, 1925

US-PAT-NO: 1533658
DOCUMENT-IDENTIFIER: US 1533658 A

TITLE: Crusher

DATE-ISSUED: April 14, 1925

INVENTOR-NAME: NEWHOUSE RAY C

US-CL-CURRENT: 241/215; 384/369

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. D

☐ 34. Document ID: US 1475600 A

L13: Entry 34 of 34 File: USOC Nov 27, 1923

US-PAT-NO: 1475600
DOCUMENT-IDENTIFIER: US 1475600 A

TITLE: Hand seed sower

DATE-ISSUED: November 27, 1923

INVENTOR-NAME: MAX SCHLING

US-CL-CURRENT: 222/544; 222/162, 222/566

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. D

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L2: Entry 29 of 29 File: PGPB Jun 28, 2001

PGPUB-DOCUMENT-NUMBER: 20010005572
PGPUB-FILING-TYPE: new-utility
DOCUMENT-IDENTIFIER: US 20010005572 A1

TITLE: Polymer overcoat for imaging elements

PUBLICATION-DATE: June 28, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Lobo, Lloyd A.	Webster	NY	US	
Nair, Mridula	Penfield	NY	US	
Lobo, Rukmini B.	Webster	NY	US	
Fitzgerald, Barry A.	Holley	NY	US	

APPL-NO: 09/ 755372 [PALM]
DATE FILED: January 5, 2001

RELATED-US-APPL-DATA:
Application 09/755372 is a division-of US application 09/311968, filed May 14, 1999, PENDING

INT-CL: [07] G03 C 1/76, G03 C 8/52, G03 C 11/06, G03 C 11/08

US-CL-PUBLISHED: 430/350; 430/207, 430/496, 430/512, 430/531, 430/533, 430/432, 430/536
US-CL-CURRENT: 430/350; 430/207, 430/432, 430/496, 430/512, 430/531, 430/533, 430/536

REPRESENTATIVE-FIGURES: NONE

ABSTRACT:

The present invention is an imaging element which includes a support, an imaging layer superposed on a side of said support and an overcoat overlying the imaging layer. The overcoat is composed of an organic polymer. The overcoat is discontinuous such that a fraction of the surface area of the imaging layer remains uncovered by said polymer, wherein the fraction of area not covered by the said polymer is from 0.02 to 0.98. The present invention is a photographic which includes a support, a silver halide emulsion layer superposed on a side of said support and an overcoat overlying the silver halide layer. The overcoat is composed of an organic polymer. The overcoat is discontinuous such that a fraction of the surface area of the silver halide emulsion layer remains uncovered by said polymer, wherein the fraction of area not covered by the said polymer is from 0.02 to 0.98. In one embodiment, the discontinuous overcoat is a series of parallel stripes.

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application relates to commonly assigned copending application Ser. No. _____, (Docket 79279) filed simultaneously and incorporated by reference herewith.

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Search Results - Record(s) 1 through 25 of 25 returned.

☐ 1. Document ID: US 6492498 B1

L11: Entry 1 of 25 File: USPT Dec 10, 2002

DOCUMENT-IDENTIFIER: US 6492498 B1
**** See image for Certificate of Correction ****
TITLE: Multimeric immunotoxins

Brief Summary Text (7):
In addition, the invention encompasses a multimeric immunotoxic protein containing at least two fusion protein monomers, each of which includes a targeting domain and a toxic domain and is physically associated with the other fusion protein monomers. The targeting domain in all the multimeric immunotoxic proteins of the invention have significant binding affinity for a target molecule on a target cell. The fusion protein monomers can contain one or more coupling moieties and the physical association of the fusion protein monomer to one or more other fusion protein monomers can be mediated by at least one of the coupling moieties. The coupling moiety can be a terminal moiety, i.e., a C-terminal moiety or a N-terminal moiety. A coupling moiety can be, for example, a cysteine residue. Furthermore the coupling moieties can be heterologous coupling moieties. The fusion protein monomers in a particular multimeric immunotoxic protein can have the same amino acid sequence or different amino acid sequences. Targeting domains can be antibody fragments, e.g., single chain Fv and can have significant binding affinity for a target molecules on a T cell, e.g., a CD3 polypeptide. Alternatively, the targeting domain can be, for example, a polypeptide such as a cytokine, a ligand for a cell adhesion receptor, a ligand for a signal transduction receptor, a hormone, a molecule that binds to a death domain family molecule (e.g., Fas ligand, TNF-alpha, or TWEAK), an antigen, or a functional fragment of any of these polypeptides. The toxic domain can be, for example, any of the following toxic polypeptides: ricin, Pseudomonas exotoxin (PE), bryodin, gelonin, .alpha.-sarcin, aspergillin, restrictocin, angiogenin, saporin, abrin, pokeweed antiviral protein (PAP), or a functional fragment of any of these toxic polypeptides. The toxic domain can also be diphtheria toxin (DT) or a functional fragment thereof, e.g., a fragment containing amino acid residues 1-389 of DT. The target cell to which the multimeric immunotoxic proteins of the invention bind can be in a mammal. The mammal can be one suspected of having graft-versus-host disease (GVHD). A target cell to which the multimeric immunotoxic proteins bind can be a cancer cell, e.g., a neural tissue cancer cell, a melanoma cell, a breast cancer cell, a lung cancer cell, a gastrointestinal cancer cell, an ovarian cancer cell, a testicular cancer cell, a lung cancer cell, a prostate cancer cell, a cervical cancer cell, a bladder cancer cell, a vaginal cancer cell, a liver cancer cell, a renal cancer cell, a bone cancer cell, and a vascular tissue cancer cell.

Detailed Description Text (22):
Toxic domains useful in the invention can be any toxic polypeptide that mediates a

cytotoxic effect on a cell. Preferred toxic polypeptides include ribosome inactivating proteins, e.g., plant toxins such as an A chain toxin (e.g., ricin A chain), saporin, bryodin, gelonin, abrin, or pokeweed antiviral protein (PAP), fungal toxins such as .alpha.-sarcin, aspergillin, or restrictocin, bacterial toxins toxins such as DT or Pseudomonas exotoxin A, or a ribonuclease such as placental ribonuclease or angiogenin. As with the targeting domains, the invention includes the use of functional fragments of any of the polypeptides. Furthermore, a particular toxic domain can include one or more (e.g., 2, 3, 4, or 6) of the toxins or functional fragments of the toxins. In addition, more than one functional fragment (e.g. 2, 3, 4, 6, 8, 10, 15, or 20) of one or more (e.g., 2, 3, 4, or 6) toxins can be included in the toxic domain. Where repeats are included, they can be immediately adjacent to each other, separated by one or more targeting fragments, or separated by a linker peptide as described above.

CLAIMS:

1. A fusion protein molecule comprising a toxic domain, a targeting domain, and at least one heterologous coupling moiety, wherein cysteine residues forming disulfide bonds within said fusion protein are: (i) cysteine residues native to the toxic domain and form disulfide bonds within the toxic domain; or (ii) cysteine residues native to the targeting domain and form disulfide bonds within the targeting domain, domain, and wherein the at least one heterologous coupling moiety is a moiety through which a second fusion protein molecule can be bound to the fusion protein molecule.

2. A multimeric immunotoxic protein comprising at least two fusion protein monomers, wherein each fusion protein monomer: comprises a targeting domain and a toxic domain; and is physically associated with the other fusion protein monomers, wherein said targeting domain binds to a target molecule on a target cell, and if a targeting domain is an antibody fragment, said antibody fragment has fewer than fourteen immunoglobulin heavy chain constant region amino acid residues, wherein an antibody fragment with no immunoglobulin heavy chain constant region amino acid residues has one VH chain and one VL chain.

3. The multimeric immunotoxic protein of claim 2, wherein each of said fusion protein monomers further comprises one or more coupling moieties and the physical association of the fusion protein monomer is by at least one of the one or more coupling moieties.

8. The multimeric immunotoxic protein of claim 2, wherein each of the fusion protein monomers comprises the same amino acid sequence.

14. The multimeric immunotoxic protein of claim 2, wherein the toxic domain is a toxic polypeptide selected from the group consisting of: (a) ricin, (b) Pseudomonas exotoxin (PE); (c) bryodin; (d) gelonin; (e) .alpha.-sarcin; (f) aspergillin; (g) restrictocin; (h) angiogenin; (i) saporin; (j) abrin; (k) pokeweed antiviral protein (PAP); and (l) a functional fragment of any of (a)-(k).

22. A multimeric immunotoxic protein comprising at least two fusion protein molecules of claim 1, each fusion protein molecule being bound by at least one of the heterologous coupling moieties to one or more of other said fusion protein molecules.

2. Document ID: US 6447777 B1

L11: Entry 2 of 25 File: USPT Sep 10, 2002

DOCUMENT-IDENTIFIER: US 6447777 B1
TITLE: Polymerized staphylococcal protein a for treatment of diseases

Detailed Description Text (26):
Most preferred superantigens are Staphylococcus aureus enterotoxins A, B, C 1, C2, D or E (SEA, SEB, SEC 1, SEC2, SED, SEE). Examples of other preferred enterotoxins or superantigens are: Streptococcus pyogenes toxins A and C (SPE-A and SPE-C; Staphylococcus aureus toxic shock syndrome-associated toxin (TSST-1); Staphylococcus aureus exfoliating toxins A and B (ETA and ETB) and Staphylococcus aureus alpha toxin.. Also included are toxins from Mycoplasma arthritides and Yersinia enterocolitica. Various enterotoxins share differing degrees of immunological relatedness (Bergdoll, M. S. et al., Infect. Immun. 4: 593 (1971); Bergdoll, M. S., Enterotoxins. In: STAPHYLOCOCCIAND STAPHYLOCOCCI INFECTIONS, C. S. F. Easmon et al., eds, pp. 559-598, 1983, London, Academic Press; Freer, J. H. et, J Pharmacol. Pharm. Ther. 19:55 (1983). Immunologic cross-reactivity between SPE-A, SEB and SEC 1 suggests the presence of a conserved domain. SEA, SEB, SEC, SED, TSST-1 and the pyrogenic exotoxins share considerable DNA and amino acid sequence homology. The enterotoxins, the pyrogenic exotoxins and TSST-1 therefore appear to be evolutionarily related and all belong to a common generic group of proteins. SPE-A and SPE-C are about as similar to each of the Staphylococcal toxins as they are to each other. Exfoliative toxins have sizes similar to SEB and SEA and similar modes of action. They share several regions of sequence similarity to the Staphylococcal enterotoxins. Overall there are several stretches of protein having similarities throughout the total group of Staphylococcal enterotoxins, Streptococcal pyrogenic exotoxins and Staphylococcal exfoliative toxins. The structural homologies between the enterotoxins and the S. pyogenes, toxins, above, apparently are responsible for the identity of clinical responses to them. These toxins induce hypotension, fever, chills and septic shock in humans, apparently by inducing cytokines such as interleukin- 1, interleukin-2, tumor necrosis factors, interferons and procoagulant activity which are the prime mediators of the clinical symptoms. Additional agents which are candidates for use in accordance with this invention in place of an enterotoxin, based upon structural homology or identity of clinical effects, are gram positive bacterial products, cell wall bacterial constituents such as peptidoglycans and various gram negative bacterial components including products of Meningococci, Pseudomonas and E. coli.

CLAIMS:

1. A composition comprising a mixture of monomeric and crosslinked polymeric protein A molecules or a functional derivative of protein A molecules, wherein (a) said cross-linked polymer molecule comprises at least two monomeric units of protein protein A or of said functional derivative; and (b) at least 10% of the total protein A or functional derivative in the form of polymers.

6. A composition according to claim 1, wherein said polymeric protein A or polymeric functional derivative molecules are complexed with immunoglobulin molecules to form polymeric protein A-immunoglobulin complexes.

7. A composition comprising a composition according to claim 6, wherein said complexes are further complexed with complement components that bind to the immunoglobulin molecules to form protein A-immunoglobulin-complement complexes.

12. A composition according to claim 11, wherein said bacterial superantigen is

selected from a group consisting of an enterotoxin of Staphylococcus aureus, toxic shock syndrome toxin, a Streptococcus pyrogenic exotoxin, a Mycoplasma arthritides toxin and a Yersinia enterocolitica toxin.

17. A method according to claim 15, wherein said crosslinking agent is selected from the group consisting of a carbodiimide, a homobifunctional aldehyde, a homobifunctional epoxide, homobifunctional imidoester, a homobifunctional N-hydroxysuccinimide ester, a homobifunctional maleimide, a homobifunctional alkyl halide, a homobifunctional pyridyl disulfide, a homobifunctional aryl halide, a homobifunctional razide, a homobifunctional diazonium derivative and a homobifunctional photoreactive compound.

22. A method for preparing a composition according to claim 11, comprising crosslinking said mixture of protein A or its functional derivative and said superantigen or its functional derivative with a carrier which carrier consists of a protein, lipid or other polymer which can be covalently bonded to said protein A or said derivative and said superantigen, thereby creating a heterogenous polymer complex, and recovering said heterogenous polymer complex.

26. A method according to claim 16, wherein said bacterial superantigen is selected from a group consisting of an enterotoxin of Staphylococcus aureus, toxic shock syndrome toxin, a Streptococcus pyrogenic exotoxin, a Mycoplasma arthritides toxin and a Yersinia enterocolitica toxin.

☐ 3. Document ID: US 6423513 B1

L11: Entry 3 of 25 File: USPT Jul 23, 2002

DOCUMENT-IDENTIFIER: US 6423513 B1
TITLE: Polynucleotides encoding protease-activatable pseudomonas exotoxin a-like proproteins

Abstract Text (1):
This invention provides protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins. The proproteins comprise (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain m of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence. The invention also provides methods of using these proproteins for killing target cells.

Brief Summary Text (2):
Methods and compositions relating to Pseudomonas exotoxin proproteins modified for selective toxicity. The exotoxin is modified to be activated by a desired protease

by insertion of a protease activatable sequence in the domain II loop. Activation of of the proprotein results in formation of the cytotoxic Pseudomonas exotoxin.

Brief Summary Text (4):
Pseudomonas Exotoxin (PE), which binds and enters mammalian cells by receptor-mediated endocytosis, depends on proteolytic cleavage to generate a C-terminal active fragment which translocates to the cell cytosol, ADP-ribosylates elongation factor 2 and inhibits protein synthesis. Mutant versions of PE which cannot be processed appropriately by cells are non-toxic. Furin has been identified as the intracellular protease responsible for this cleavage. Cleavage occurs between arginine 279 and glycine 280 in an arginine-rich loop located in domain II of the toxin. In biochemical experiments, furin-mediated cleavage is evident only under mildly acidic conditions (pH 5.5). Recently, Garten et al., (EMBO J, 14(11):2424-35 (1995)) have proposed that sequences in the cytoplasmic tail of furin are responsible for its cycling to the cell surface and reentry through the endosomal compartnent. Since PE enters cells via the alpha 2-macroglobulin receptor/Low density lipoprotein receptor-related protein (LRP), it is likely that this receptor delivers PE to an acidic endosomal compartment where it is cleaved by furin. PE is broadly cytotoxic because most mammalian cells and tissues express both LRP and furin. In vivo, the injection of native PE produces profound liver toxicity.

Brief Summary Text (10):
Pseudomonas Exotoxin A ("PE") is translocated into the cytosol after a furin recognition site in domain II is cleaved by furin. Protease-activatable PE-like proproteins are engineered to replace the furin recognition site by a site recognized by a protease made or secreted by a cell targeted for death, for example, a cancer cell. Upon cleavage by the target protease, the PE-like proprotein is translocated into the cytosol where the toxin's ADP-ribosylating activity kills the cell by interfering with polypeptide elongation.

Brief Summary Text (14):
In one aspect this invention provides a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence.

Brief Summary Text (16):
In another aspect, this invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention.

Brief Summary Text (17):
In another aspect, this invention provides a recombinant polynucleotide comprising a nucleotide sequence encoding a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the recombinant polynucleotide is an expression vector further comprising an expression control sequence operatively linked to the nucleotide sequence.

Brief Summary Text (18):
In another aspect, this invention provides a method for killing a target cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like

("PE-like") proprotein of this invention. In one embodiment, the cancer cell is, without limitation, a prostate cancer cell, a breast cancer cell or a colon cancer cell.

Brief Summary Text (19):
In another aspect, this invention provides a method for therapeutically treating a subject suffering from cancer comprising administering to the subject a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. More specifically, the PE-like proprotein comprises a protease activatable sequence that is cleavable by an enzyme produced by the cancer cell. The PE-like proprotein can be administered as a pharmaceutical composition.

Drawing Description Text (17):
FIG. 8 is a diagram of Pseudomonas Exotoxin A structure. The amino acid position based on SEQ ID NO:2 is indicated. Domain 1a extends from amino acids 1-252. Domain II extends from amino acids 253-364. It includes a cysteine-cysteine loop formed by cysteines at amino acids 265-287. Furin cleaves within the cysteine-cysteine loop between amino acids 279 and 280. A fragment of PE beginning with amino acid 280 translocates to the cytosol. Constructs in which amino acids 345-364 are eliminated also translocate. Domain Ib spans amino acids 365-399. It contains a cysteine-cysteine loop formed by cysteines at amino acids 372 and 379. The domain can be eliminated entirely. Domain III spans amino acids 400-613. Deletion of amino acid 553 eliminates ADP ribosylation activity. The endoplasmic reticulum sequence, REDKL (SEQ ID NO:33) is located at the carboxy-terminus of the molecule, from amino acid 609-613.

Detailed Description Text (45):
"Pseudomonas exotoxin A" or "PE" is secreted by P. aeruginosa as a 67 kD protein composed of three prominent globular domains (Ia, II, and III) and one small subdomain (Ib) connecting domains II and III. (A. S. Allured et al. (1986) Proc. Natl. Acad. Sci. 83:1320-1324.) Domain Ia of PE mediates cell binding. In nature, domain Ia binds to the low density lipoprotein receptor-related protein ("LRP"), also known as the .alpha.2-macroglobulin receptor (".alpha.2-MR"). (M. Z. Kounnas et al. (1992) J. Biol. Chem. 267:12420-23.). It spans amino acids 1-252. Domain II mediates translocation to the cytosol. It spans amino acids 253-364. Domain Ib has no identified function. It spans amino acids 365-399. Domain III is responsible for cytotoxicity and includes an endoplasmic reticulum retention sequence. It mediates ADP ribosylation of elongation factor 2, which inactivates protein synthesis. It spans amino acids 400-613. PE is "detoxified" if it lacks EF2 ADP ribosylation activity. Deleting amino acid E553 (".DELTA.E553") from domain III detoxifies the molecule. PE having the mutation AE553 is referred to herein as "PE .DELTA.E553." Genetically modified forms of PE are described in, e.g., Pastan et al., U.S. Pat. No. 5,602,095; Pastan et al., U.S. Pat. No. 5,512,658, Pastan et al., U.S. Pat. No. 5,458,878 and Pastan et al., U.S. Pat. No. 5,328,984. Allelic forms of PE are included in this definition. See, e.g., M. L. Vasil et al., (1986) Infect. Immunol. 52:538-48. The nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of Pseudomonas exotoxin A are:

Detailed Description Text (46):
PROTEASE-ACTIVATABLE PSEUDOMONAS EXOTOXIN A-LIKE PROPROTEINS

Detailed Description Text (48):
Protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins are polypeptides having structural domains organized, except as provided herein, in the same general sequence as the four structural domains of PE, and having certain functions (e.g., cell recognition, cytosolic translocation, cytotoxicity and endoplasmic reticulum retention) also possessed by the functional domains of PE. More specifically, the general order is: domain Ia, domain II, domain Ib, domain III. However, as described in more detail herein, domain Ia can be eliminated and replaced by a binding protein chemically coupled to the molecule, or, a cell

recognition domain can be inserted just before the ER retention sequence in domain III. Domain Ib can be eliminated. Domain II is positioned to the amino-terminal side side of domain III.

Detailed Description Text (53):
Protease-activatable Pseudomonas exotoxin-like proproteins comprise an amino acid sequence encoding a "cell recognition domain." The cell recognition domain functions as a ligand for a cell surface receptor. It mediates binding of the protein to a cell. Its purpose is to target the proprotein to a cell which will transport the proprotein to the cytosol for processing. The cell recognition domain can be located in the position of domain Ia of PE. However, this domain can be moved out of the normal organizational sequence. More particularly, the cell recognition domain can be inserted upstream of the ER retention sequence. Alternatively the cell recognition domain can be chemically coupled to the toxin. Also, the proprotein can include a first cell recognition domain at the location of the Ia domain and a second cell recognition domain upstream of the ER retention domain. Such constructs can bind to more than one cell type. See, e.g., R. J. Kreitman et al., Blood 90, pp. 252-259 (1992) Bioconjugate Chem. 3:63-68.

Detailed Description Text (57):
In one embodiment, the cell recognition domain is located in place of domain Ia of PE. In another embodiment, it can be attached to the other moiety of the molecule through a linker. Engineering studies also show that Pseudomonas exotoxin can be targeted to certain cell types by introducing a cell recognition domain upstream of the ER retention sequence, which is located at the carboxy-terminus of the polypeptide. For example, TGF.alpha. has been inserted into domain III just before amino acid 604, i.e., about ten amino acids from the carboxy-terminus. This chimeric protein binds to cells bearing EGF receptor. Pastan et al., U.S. Pat. No. 5,602,095.

Detailed Description Text (80):
In native Pseudomonas exotoxin A, domain Ib spans amino acids 365 to 399. The native native Ib domain is structurally characterized by a disulfide bond between two cysteines at positions 372 and 379. Domain Ib is not essential for cell binding, translocation, ER retention or ADP ribosylation activity. Therefore, it can be entirely re-engineered or eliminated completely. The PE 1b-like domain can be linear or it can include a cysteine-cysteine loop.

Detailed Description Text (200):
The present invention provides Pseudomonas exotoxin A-like proproteins and methods of using them. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

Detailed Description Paragraph Table (4):
SEQUENCE LISTING (1) GENERAL INFORMATION: (iii) NUMBER OF SEQUENCES: 36 (2) INFORMATION FOR SEQ ID NO: 1: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1839 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1..1839 (D) OTHER INFORMATION: /product= "Pseudomonas exotoxin A" (xi) SEQUENCE DESCRIPTION: DESCRIPTION: SEQ ID NO:1 GCC GAA GAA GCT TTC GAC CTC TGG AAC GAA TGC GCC AAA GCC TGC GTG 48 Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10 15 CTC GAC CTC AAG GAC GGC GTG CGT TCC AGC CGC ATG AGC GTC GAC CCG 96 Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp Pro 20 25 30 GCC ATC GCC GAC ACC AAC GGC CAG GGC GTG CTG CAC TAC TCC ATG GTC 144 Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met Val 35 40 45 CTG GAG GGC GGC AAC GAC GCG CTC AAG CTG GCC ATC GAC AAC GCC CTC 192 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala

Leu 50 55 60 AGC ATC ACC AGC GAC GGC CTG ACC ATC CGC CTC GAA GGC GGC GTC GAG 240 Ser
Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val Glu 65 70 75 80 CCG AAC
AAG CCG GTG CGC TAC AGC TAC ACG CGC CAG GCG CGC GGC AGT 288 Pro Asn Lys Pro Val Arg
Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85 90 95 TGG TCG CTG AAC TGG CTG GTA CCG ATC
ATC GGC CAC GAG AAG CCC TCG AAC 336 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu
Lys Pro Ser Asn 100 105 110 ATC AAG GTG TTC ATC CAC GAA CTG AAC GCC GGC AAC CAG CTC
AGC CAC 384 Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120
125 ATG TCG CCG ATC TAC ACC ATC GAG ATG GGC GAC GAG TTG CTG GCG AAG 432 Met Ser Pro
Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 CTG GCG CGC GAT GCC
ACC TTC TTC GTC AGG GCG CAC GAG AGC AAC GAG 480 Leu Ala Arg Asp Ala Thr Phe Phe Val
Arg Ala His Glu Ser Asn Glu 145 150 155 160 ATG CAG CCG ACG CTC GCC ATC AGC CAT GCC
GGG GTC AGC GTG GTC ATG 528 Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val
Val Met 165 170 175 GCC CAG ACC CAG CCG CGC CGG GAA AAG CGC TGG AGC GAA TGG GCC AGC
576 Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 GGC
AAG GTG TTG TGC CTG CTC GAC CCG CTG GAC GGG GTC TAC AAC TAC 624 Gly Lys Val Leu Cys
Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr 195 200 205 CTC GCC CAG CAA CGC TGC AAC
CTC GAC GAT ACC TGG GAA GGC AAG ATC 672 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr
Trp Glu Gly Lys Ile 210 215 220 TAC CGG GTG CTC GCC GGC AAC CCG GCG AAG CAT GAC CTG
GAC ATC AAA 720 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys 225
230 235 240 CCC ACG GTC ATC AGT CAT CGC CTG CAC TTT CCC GAG GGC GGC AGC CTG 768 Pro
Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser Leu 245 250 255 GCC GCG CTG
ACC GCG CAC CAG GCT TGC CAC CTG CCG CTG GAG ACT TTC 816 Ala Ala Leu Thr Ala His Gln
Ala Cys His Leu Pro Leu Glu Thr Phe 260 265 270 ACC CGT CAT CGC CAG CCG CGC GGC TGG
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Gln Cys Gly 275 280 285 TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC CTG GCG GCG CGG CTG
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GGC GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC 1008 Ser Gly Gly Asp Leu Gly Glu Ala Ile
Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335 CGT CTG GCC CTG ACC CTG GCC GCC GCC GAG
AGC GAG CGC TTC GTC CGG 1056 Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe
Phe Val Arg 340 345 350 CAG GGC ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG
GTG 1104 Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val 355 360 365
365 AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG GCG GAC 1152 Ser Leu Thr
Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp 370 375 380 AGC GGC GAC GCC
CTG CTG GAG CGC AAC TAT CCC ACT GGC GCG GAG TTC 1200 Ser Gly Asp Ala Leu Leu Glu Arg
Arg Asn Tyr Pro Thr Gly Ala Glu Phe 385 390 395 400 CTC GGC GAC GGC GGC GAC GTC AGC
TTC AGC ACC CGC GGC ACG CAG AAC 1248 Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg
Arg Gly Thr Gln Asn 405 410 415 TGG ACG GTG GAG CGG CTG CTC CAG GCG CAC CGC CAA CTG
GAG GAG CGC 1296 Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg 420
420 425 430 GGC TAT GTG TTC GTC GGC TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA 1344 Gly
Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln 435 440 445 AGC ATC
GTC TTC GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG 1392 Ser Ile Val Phe Gly Gly
Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala 450 455 460 ATC TGG CGC GGT TTC TAT ATC
GCC GGC GAT CCG GCG CTG GCC TAC GGC 1440 Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro
Pro Ala Leu Ala Tyr Gly 465 470 475 480 TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC
CGG ATC CGC AAC GGT 1488 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn
Asn Gly 485 490 495 GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG CCG GGC TTC TAC
1536 Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr 500 505 510 CGC
CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG GCG GCG GGC GAG GTC GAA 1584 Arg Thr Ser Leu
Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu 515 520 525 CGG CTG ATC GGC CAT
CCG CTG CCG CTG CGC CTG GAC GCC ATC ACC GGC 1632 Arg Leu Ile Gly His Pro Leu Pro Leu
Leu Arg Leu Asp Ala Ile Thr Gly 530 535 540 CCC GAG GAG GAA GGC GGG CGC CTG GAG ACC
ATT CTC GGC TGG CCG CTG 1680 Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp
Trp Pro Leu 545 550 555 560 GCC GAG CGC ACC GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC
CCG CGC 1728 Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg 565 570
570 575 AAC GTC GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1776 Asn Val
Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln 580 585 590 GCG ATC AGC
GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA CCG CCG 1824 Ala Ile Ser Ala Leu Pro Asp
Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro 595 600 605 CGC GAG GAC CTG AAG 1839 Arg Glu

Glu Asp Leu Lys 610 (2) INFORMATION FOR SEQ ID NO: 2: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 613 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2 Ala Glu Glu Ala Phe Asp Leu Trp Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10 15 Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp Pro 20 25 30 Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His His Tyr Ser Met Val 35 40 45 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Asn Ala Leu 50 55 60 Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val Glu Glu 65 70 75 80 Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85 90 95 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser Asn 100 105 110 Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120 125 Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn Glu 145 150 155 160 Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val Met 165 170 175 Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr 195 200 205 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys Ile 210 215 220 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys 225 230 235 240 Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser Leu 245 250 255 Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe 260 265 270 Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly 275 280 285 Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser 290 295 300 Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly 305 310 315 320 Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335 Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg 340 345 350 Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val 355 360 365 Ser Leu Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp 370 375 380 Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe 385 390 395 400 Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn 405 410 415 Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg 420 425 430 Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln 435 440 445 Ser Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala 450 455 460 Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly 465 470 475 480 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly 485 490 495 Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr 500 505 510 Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu 515 520 525 Arg Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly 530 535 540 Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu 545 550 555 560 Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg 565 570 575 Asn Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln 580 585 590 Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro 595 600 605 Arg Glu Asp Leu Lys 610 (2) INFORMATION FOR SEQ ID NO: 3: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 8 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3 Asp Arg Val Tyr Ile His Pro Phe 1 5 (2) INFORMATION FOR SEQ SEQ ID NO: 4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 12 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4 Val Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro 1 5 10 (2) INFORMATION FOR SEQ ID NO: 5: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 7 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5 Phe Phe Tyr Thr Pro Lys Ala 1 5 (2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 9 amino acids

Other Reference Publication (4):
Allured et al., "Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution," Proc. Nat'l Acad. Sci., 83:1320-1324 (1986).

Other Reference Publication (7):
Pastan et al., "Pseudomonas exotoxin: Chimeric toxins," J. of Biol. Chem., 264 (26):15157-15160 (1989).

CLAIMS:

1. A recombinant polynucleotide comprising a nucleotide sequence encoding a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain of between 10 and 1500 amino acids that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

2. The recombinant polynucleotide of claim 1, further comprising a nucleic acid sequence encoding a PE Ib-like domain comprising an amino acid sequence of between 5 5 and about 1500 amino acids, which amino acid sequence is positioned between the modified PE translocation domain and the cytotoxicity domain and which does not interfere with the ability of the PE-like proprotein to bind cells, translocate, or ribosylate ADP.

3. The recombinant polynucleotide of claim 1 which is an expression vector further comprising an expression control sequence operatively linked to the nucleotide sequence.

4. The recombinant polynucleotide of claim 2 wherein: (a) the cell recognition domain is an antibody coupled to the modified PE translocation domain through a peptide bond and wherein the antibody specifically binds a cancer cell surface marker; (b) the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO:2) modified with amino acid substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280: and (c) the PE Ib-like domain, the cytotoxicity domain and the ER retention sequence together have the sequence of domains Ib and III of native PE.

5. The recombinant polynucleotide of claim 4 wherein the protease activatable sequence is cleavable by prostate specific antigen or urokinase.

☐ 4. Document ID: US 6337194 B1

L11: Entry 4 of 25 File: USPT Jan 8, 2002

DOCUMENT-IDENTIFIER: US 6337194 B1
TITLE: Method for the preparation of insulin by cleavage of a fusion protein and fusion proteins containing insulin A and B chains

Brief Summary Text (33):
N may be aspartic acid and treatment with for example Pseudomonas fragi mutant Me1 endopeptidase may cause cleavage of the purification sequence from the second polypeptide.

Brief Summary Text (53):

Alternatively, such a method of production may comprise transforming the host organism with an expression vector encoding a protein precursor wherein Z is of the general formula (VI), cultivating the transformed host in a suitable culture medium, medium, recovering the secreted product, converting it to mature insulin by cleavage cleavage at the aspartic acid residue by Pseudomonas fragi Me1 endopeptidase treatment in order to remove the chain of amino acid residues X. The chain of amino acid residues X may comprise at least either a purification sequence or a second polypeptide.

Detailed Description Text (55):
Precursors having the general formula (VI): B-N-X-KR-A are cleaved in vivo by KEX2 and secreted by yeast in the form: A::B-N-X(-KR) where (-KR) indicates that these residues may be removed by KEX1 activity in vivo or by carboxypeptidase B in vitro. If N is aspartic acid, in vitro treatment with P. fragi mutant Me1 endopeptidease (Sigma Catalogue No. P3303 Me1 endopeptidase from mutant Pseudomonas fragi) cleaves amino-terminally to the N residue to produce mature insulin.

CLAIMS:

6. A DNA sequence encoding a single-chain insulin precursor having the formula B-KR-Pur-M-A wherein A and B represent the A and B polypeptide chains, respectively, of insulin, K is lysine, R is arginine, Pur is a purification sequence of amino acid residues that can be recognized and bound by another molecule for in vitro separation of an insulin precursor containing the sequence from a mixture of molecules, and M is methionine, and cleavage at the KR residues produces a double-chain insulin precursor Pur-M-A::B, wherein :: represents two disulfide bonds established between the A and B chains.

10. A DNA sequence encoding a single-chain insulin precursor having the formula B-KR-Y-M-A wherein A and B represent the A and B polypeptide chains, respectively, of insulin, K is lysine, R is arginine, Y is an additional biological polypeptide of interest, and M is methionine, and cleavage at the KR residues produces a double-chain insulin precursor Y-M-A::B, wherein :: represents two disulfide bonds established between the A and B chains.

14. A DNA sequence encoding a single-chain insulin precursor having the formula B-KR-Y-N-Pur-M-A, wherein A and B represent the A and B polypeptide chains, respectively, of insulin, K is lysine, R is arginine, Y is an additional biological polypeptide of interest, N is methionine or aspartic acid, Pur is a purification sequence of amino acid residues that can be recognized and bound by another molecule for in vitro separation of an insulin precursor containing the sequence from a mixture of molecules, and M is methionine, and cleavage at the KR residues produces a double-chain insulin precursor Y-N-Pur-M-A::B, wherein :: represents two disulfide bonds established between the A and B chains.

16. The DNA sequence according to claim 14 wherein N is aspartic acid and treatment of the double-chain insulin precursor with Pseudomonas fragi mutant Me1 endopeptidase causes cleavage of the polypeptide Y from the purification sequence.

19. A DNA sequence encoding a single-chain insulin precursor having the formula B-N-X-KR-A wherein A and B represent the A and B polypeptide chains, respectively, of insulin, N is methionine or aspartic acid, K is lysine, R is arginine, and X is a chain of amino acid residues sufficient in length to facilitate cleavage in a yeast or fungal host cell at the KR residues, and cleavage at the KR residues produces a double-chain insulin precursor A::B-N-X wherein :: represents two disulfide bonds established between the A and B chains.

24. A double-chain insulin precursor having the formula Pur-M-A::B wherein A and B represent the A and B polypeptide chains, respectively, of insulin and :: represents two disulfide bonds established between the A and B chains, M is

methionine, and Pur is a purification sequence of amino acid residues that can be recognized and bound by another molecule for in vitro separation of the insulin precursor from a mixture of molecules.

25. The double-chain insulin precursor according to claim 24 wherein the double-chain precursor is secreted by a yeast or fungus host cell transformed by a recombinant DNA coding for a single-chain precursor of the double-chain insulin precursor and a leader peptide.

28. A double-chain insulin precursor having the formula Y-M-A::B wherein A and B represent the A and B polypeptide chains, respectively, of insulin and :: represents two disulfide bonds established between the A and B chains, M is methionine, and Y is an additional polypeptide of interest.

29. The double-chain insulin precursor according to claim 28 wherein the double-chain precursor is secreted by a yeast or fungus host cell transformed by a recombinant DNA coding for a single-chain precursor of the double-chain insulin precursor and a leader peptide.

32. A double-chain insulin precursor having the formula Y-N-Pur-M-A::B wherein A and B represent the A and B polypeptide chains, respectively, of insulin and :: represents two disulfide bonds established between the A and B chains, M is methionine, N is methionine or aspartic acid, Y is an additional polypeptide of interest, and Pur is a purification sequence of amino acid residues that can be recognized and bound by another molecule for in vitro separation of the insulin precursor from a mixture of molecules.

33. The double-chain insulin precursor according to claim 32 wherein the double-chain precursor is secreted by a yeast or fungus host cell transformed by a recombinant DNA coding for a single-chain precursor of the double-chain insulin precursor and a leader peptide.

36. A double-chain insulin precursor having the formula A::B-N-X wherein A and B represent the A and B polypeptide chains, respectively, of insulin and :: represents two disulfide bonds established between the A and B chains, N is methionine or aspartic acid, and X is a chain of amino acid residues that comprises a purification sequence Pur and/or an additional polypeptide of interest, wherein Pur is a sequence of amino acid residues that can be recognized and bound by another molecule for in vitro separation of an insulin precursor containing the sequence from a mixture of molecules.

37. The double-chain insulin precursor according to claim 36 wherein the double-chain precursor is secreted by a yeast or fungus host cell transformed by a recombinant DNA coding for a single-chain precursor of the double-chain insulin precursor and a leader peptide.

40. A method for preparing insulin from a single-chain protein precursor having the general formula B-Z-A wherein B and A are the two polypeptide chains representing, respectively, the B- and A- chains of insulin, and Z is a polypeptide comprising at least one site for proteolytic cleavage in a transformed yeast or fungus host cell, the method comprising the steps of:

transforming or transfecting a eukaryotic host cell with an expression vector expressing a DNA sequence encoding a single-chain protein precursor of insulin and comprising any one of SEQ ID NOs: 10 to 18, or a DNA sequence that encodes the same protein as encoded by any one of SEQ ID NOs: 10 to 18, and a leader peptide which directs the protein precursor into a secretion pathway of the host cell;

cultivating the transformed host in a suitable culture medium;

recovering from the culture medium a secreted double-chain insulin precursor having the formula -A::B or A::B- or -A::B-, resulting from proteolytic cleavage of the single-chain precursor by the host cell, wherein :: represents two disulfide bonds established between the A and B chains and "--" represents a retained portion of the Z polypeptide on the A and/or the B chain; and

converting the secreted double-chain insulin precursor to insulin by treatment with a cleaving agent to remove the retained portions of the Z polypeptide.

51. A method according to claim 49 wherein the converting step includes cleaving the double-chain insulin precursor at the aspartic acid residue N by Pseudomonas fragi Me1 endopeptidase treatment to remove the X chain of amino acid residues.

5. Document ID: US 6177615 B1

L11: Entry 5 of 25 File: USPT Jan 23, 2001

DOCUMENT-IDENTIFIER: US 6177615 B1
TITLE: Lepidopteran-toxic polypeptide and polynucleotide compositions and methods for making and using same

Brief Summary Text (165):
In one preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension comprising lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferably the cells are B. thuringiensis cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein is contemplated to be useful, such as Bacillus spp., including B. megaterium, B. subtilis; B. cereus, Escherichia spp., including E. coli, and/or Pseudomonas spp., including P. cepacia, P. aeruginosa, and P. fluorescens. Alternatively, the oil flowable suspension may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (166):
In a second preferred embodiment, the bioinsecticide composition comprises a water dispersible granule or powder. This granule or powder may comprise lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferred sources for these compositions include bacterial cells such as B. thuringiensis cells, however, bacteria of the genera Bacillus, Escherichia, and Pseudomonas which have been transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Alternatively, the granule or powder may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (167):
In a third important embodiment, the bioinsecticide composition comprises a wettable powder, spray, emulsion, colloid, aqueous or organic solution, dust, pellet, or collodial concentrate. Such a composition may contain either unlysed or lysed bacterial cells, spores, crystals, or cell extracts as described above, which

contain one or more of the novel crystal proteins disclosed herein. Preferred bacterial cells are B. thuringiensis cells, however, bacteria such as B. megaterium, megaterium, B. subtilis, B. cereus, E. coli, or Pseudomonas spp. cells transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or alternatively, dissolve in a controlled-release, sustained-release, or other time-dependent manner. Alternatively, such a composition may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Detailed Description Text (16):
The nucleotide sequences of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the sites of lepidopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B. thuringiensis toxin.

Detailed Description Text (17):
Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility or toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and Gram-positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium; Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae, Actinomycetales, and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

Detailed Description Text (19):
Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Streptomyces lividans and the like.

Detailed Description Text (22):
A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera Bacillus, Pseudomonas, Erwinia, Serratia, Klebsiella, Zanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes; fungi,

particularly yeast, e.g., genera Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phytosphere bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodobacter sphaeroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes eutrophus, and Azotobacter vinlandii; and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans.

Detailed Description Text (54):
In an alternate embodiment, the recombinant expression of DNAs encoding the crystal proteins of the present invention is performed using a transformed Gram-negative bacterium such as an E. coli or Pseudomonas spp. host cell. Promoters which function function in high-level expression of target polypeptides in E. coli and other Gram-negative host cells are also well-known in the art.

CLAIMS:

1. An isolated B. thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry1C .delta.-endotoxin polypeptide.

2. The polypeptide of claim 1, wherein said loop region extends from about amino acid 176 to about amino acid 185 of a native Cry1C .delta.-endotoxin polypeptide.

10. A composition comprising a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

17. A method of preparing a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry1 .delta.-endotoxin polypeptide, comprising

(a) identifying a Cry1 .delta.-endotoxin polypeptide having a loop region between .alpha. helices 5 and 6 of domain 1 of said polypeptide;

(b) substituting at least one native amino acid in said loop region with another amino acid; and

(c) obtaining the Cry1 .delta.-endotoxin polypeptide so produced.

21. The method of claim 17, wherein said loop region extends from about amino acid 176 to about amino acid 185 of a native Cry1C polypeptide.

25. A polynucleotide comprising an isolated gene that encodes a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry1C .delta.-endotoxin polypeptide.

28. The polynucleotide of claim 25, wherein said loop region extends from about amino acid 176 to about amino acid 185 of the native Cry1C .delta.-endotoxin polypeptide.

34. The polynucleotide of claim 25, wherein said gene is operably linked to a promoter that expresses said gene to produce said polypeptide.

37. The polynucleotide of claim 36, wherein said plant-expressible promoter is selected from the group consisting of corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, pea small subunit RuBP carboxylase, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, Potato patatin, lectin, CaMV 35S, and the S-E9 small subunit RuBP carboxylase promoter.

38. A vector comprising at least one gene that encodes a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry 1C .delta.-endotoxin polypeptide.

42. A host cell comprising a gene encoding a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry1C .delta.-endotoxin polypeptide.

45. The host cell of claim 44, wherein said bacterial cell is an E. coli, Bacillus thuringiensis, Bacillus subtilis, Bacillus megaterium, Bacillus cereus or Pseudomonas spp. cell.

50. A transgenic plant having incorporated into its genome a selected polynucleotide, said polynucleotide comprising a gene that encodes a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

51. The transgenic plant of claim 50, wherein said loop region extends from about amino acid 176 to about amino acid 185 of the native Cry1C polypeptide.

65. A method of controlling Lepidopteran insects comprising contacting said insects with an insecticidally-effective amount of a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having at least one amino acid mutation in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry 1C .delta.-endotoxin polypeptide.

69. A method of killing a Lepidopteran insect comprising feeding to said insect an insecticidally-effective amount of a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having at least one amino acid mutation in the loop region between .alpha. helices 5 and 6 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cry1C .delta.-endotoxin polypeptide.

☐ 6. Document ID: US 6153814 A

L11: Entry 6 of 25 File: USPT Nov 28, 2000

DOCUMENT-IDENTIFIER: US 6153814 A
TITLE: Polypeptide compositions toxic to lepidopteran insects and methods for making making same

Brief Summary Text (162):
In one preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension comprising lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferably the cells are B. thuringiensis cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein is contemplated to be useful, such as Bacillus spp., including B. megaterium, B. subtilis; B. cereus, Escherichia spp., including E. coli, and/or Pseudomonas spp., including P. cepacia, P. aeruginosa, and P. fluorescens. Alternatively, the oil flowable suspension may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (163):
In a second preferred embodiment, the bioinsecticide composition comprises a water dispersible granule or powder. This granule or powder may comprise lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferred sources for these compositions include bacterial cells such as B. thuringiensis cells, however, bacteria of the genera Bacillus, Escherichia, and Pseudomonas which have been transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Alternatively, the granule or powder may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (164):
In a third important embodiment, the bioinsecticide composition comprises a wettable powder, spray, emulsion, colloid, aqueous or organic solution, dust, pellet, or collodial concentrate. Such a composition may contain either unlysed or lysed bacterial cells, spores, crystals, or cell extracts as described above, which contain one or more of the novel crystal proteins disclosed herein. Preferred bacterial cells are B. thuringiensis cells, however, bacteria such as B. megatenum, B. subtilis, B. cereus, E. coli, or Pseudomonas spp. cells transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or alternatively, dissolve in a controlled-release, sustained-release, or other time-dependent manner. Alternatively, such a composition may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Detailed Description Text (16):
The nucleotide sequences of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the sites of lepidopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B. thuringiensis toxin.

Detailed Description Text (17):
Suitable host cells, where the pesticide-containing cells will be treated to

prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic toxic to higher organisms could be used, where the toxin is unstable or the level of of application sufficiently low as to avoid any possibility or toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and Gram-positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium; Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae, Actinomycetales, and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidiun, Sporobolomyces, and the like.

Detailed Description Text (19):
Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Streptomyces lividans and the like.

Detailed Description Text (22):
A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g. genera Bacillus, Pseudomonas, Ewinia, Serratia, Klebsiella, Zanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes; fungi, particularly yeast, e.g., genera Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phytosphere bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodobacter sphaeroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes eutrophus, and Azotobacter vinlandii; and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans.

Detailed Description Text (54):
In an alternate embodiment, the recombinant expression of DNAs encoding the crystal proteins of the present invention is performed using a transformed Gram-negative bacterium such as an E. coli or Pseudomonas spp. host cell. Promoters which function function in high-level expression of target polypeptides in E. coli and other Gram-negative host cells are also well-known in the art.

CLAIMS:

1. An isolated B. thuringiensis CrylC .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native CrylC .delta.-endotoxin polypeptide.

2. The polypeptide of claim 1, wherein said loop region extends from about amino

acid 118 to about amino acid 124 of a native CrylC .delta.-endotoxin polypeptide.

18. A composition comprising a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native CrylC .delta.-endotoxin polypeptide.

25. A method of preparing a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native Cryl .delta.-endotoxin polypeptide, comprising

(a) identifying a Cryl .delta.-endotoxin polypeptide having a loop region between a helices 3 and 4 of domain 1 of said polypeptide;

(b) substituting at least one native amino acid in said loop region with another amino acid; and

(c) obtaining the Cryl .delta.-endotoxin polypeptide so produced.

29. The method of claim 25, wherein said loop region extends from about amino acid 118 to about amino acid 124 of a native CrylC polypeptide.

34. A polynucleotide comprising an isolated gene that encodes a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native CrylC .delta.-endotoxin polypeptide.

35. The polynucleotide of claim 34, wherein said loop region extends from about amino acid 118 to about amino acid 124 of the native CrylC .delta.-endotoxin polypeptide.

49. The polynucleotide of claim 34, wherein said gene is operably linked to a promoter that expresses said gene to produce said polypeptide.

52. The polynucleotide of claim 51, wherein said plant-expressible promoter is selected from the group consisting of corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, pea small subunit RuBP carboxylase, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, Potato patatin, lectin, CaMV 35S, and the S-E9 small subunit RuBP carboxylase promoter.

53. A vector comprising at least one gene that encodes a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native CrylC .delta.-endotoxin polypeptide.

57. A host cell comprising a gene encoding a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native CrylC .delta.-endotoxin polypeptide.

60. The host cell of claim 59, wherein said bacterial cell is an E. coli, Bacillus thuringiensis, Bacillus subtilis, Bacillus megaterium, Bacillus cereus or Pseudomonas spp. cell.

65. A transgenic plant having incorporated into its genome a selected

polynucleotide, said polynucleotide comprising a gene that encodes a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native CrylC .delta.-endotoxin polypeptide.

66. The transgenic plant of claim 65, wherein said loop region extends from about amino acid 118 to about amino acid 124 of the native CrylC polypeptide.

86. A method of controlling Lepidopteran insects comprising contacting said insects with an insecticidally-effective amount of a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having at least one amino acid mutation in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to native CrylC .delta.-endotoxin polypeptide.

89. A method of killing a Lepidopteran insect comprising feeding to said insect an insecticidally-effective amount of a Bacillus thuringiensis CrylC .delta.-endotoxin polypeptide having at least one amino acid mutation in the loop region between .alpha. helices 3 and 4 of domain 1, said polypeptide having improved insecticidal activity against said Lepidopteran insect when compared to native CrylC .delta.-endotoxin polypeptide.

☐ 7. Document ID: US 6127607 A

L11: Entry 7 of 25 File: USPT Oct 3, 2000

DOCUMENT-IDENTIFIER: US 6127607 A
TITLE: Plant resistance gene family encoding resistance polypeptides having P-loop and LRR motifs

Detailed Description Text (4):
One basic prediction of the gene-for-gene hypothesis has been convincingly confirmed at the molecular level by the cloning of a variety of bacterial avr genes (Innes, et al., (1993) J. Bacteriol. 175:4859-4869; Dong, et al., (1991) Plant Cell 3:61-72; Whelan et al., (1991) Plant Cell 3:49-59; Staskawicz et al., (1987) J. Bacteriol. 169:5789-5794; Gabriel et al., (1986) P.N.A.S., USA 83:6415-6419; Keen and Staskawicz, (1988) Annu. Rev. Microbiol. 42:421-440; Kobayashi et al., (1990) Mol. Plant-Microbe Interact. 3:94-102 and (1990) Mol. Plant-Microbe Interact. 3:103-111). Many of these cloned avirulence genes have been shown to correspond to individual resistance genes in the cognate host plants and have been shown to confer an avirulent phenotype when transferred to an otherwise virulent strain. The avrRpt2 locus was isolated from Pseudomonas syringae pv. tomato and sequenced by Innes et al. (Innes, R. et al. (1993) J. Bacteriol. 175:4859-4869). FIG. 3 is the nucleotide sequence (SEQ ID NO: 105) and deduced amino acid sequence (SEQ ID NO: 106) of the avrRpt2 gene.

Detailed Description Text (5):
Examples of known signals to which plants respond when infected by pathogens include harpins from Erwinia (Wei et al. (1992) Science 257:85-88) and Pseudomonas (He et al. (1993) Cell 73:1255-1266); avr4 (Joosten et al. (1994) Nature 367:384-

386) and avr9 peptides (van den Ackerveken et al (1992) Plant J. 2:359-366) from Cladosporium; PopA1 from Pseudomonas (Arlat et al. (1994) EMBO J. 13:543-553); avrD-avrD-generated lipopolysaccharide (Midland et al. (1993) J. Org. Chem. 58:2940-2945); and NIP1 from Rhynchosporium (Hahn et al. (1993) Mol. Plant-Microbe Interact. 6:745-754).

Detailed Description Text (6):
Compared to avr genes, considerably less is known about plant resistance genes that correspond to specific avr-generated signals. The plant resistance gene, RPS2 (rps for resistance to Pseudomonas syringae), the first gene of a new, previously unidentified class of plant disease resistance genes corresponds to a specific avr gene (avrRpt2). Some of the work leading up to the cloning of RPS2 is described in Yu, et al., (1993), Molecular Plant-Microbe Interactions 6:434-443 and in Kunkel, et al., (1993) Plant Cell 5:865-875.

Detailed Description Text (7):
An apparently unrelated avirulence gene which corresponds specifically to plant disease resistance gene, Pto, has been isolated from tomato (Lycopersicon esculentum) (Martin et al., (1993) Science 262:1432-1436). Tomato plants expressing the Pto gene are resistant to infection by strains of Pseudomonas syringae pv. tomato that express the avrPto avirulence gene. The amino acid sequence inferred from the Pto gene DNA sequence displays strong similarity to serine-threonine protein kinases, implicating Pto in signal transduction. No similarity to the tomato Pto locus or any known protein kinases was observed for RPS2, suggesting that RPS2 is representative of a new class of plant disease resistance genes.

Detailed Description Text (12):
To demonstrate the genetic relationship between an avirulence gene in the pathogen and a resistance gene in the host, it was necessary first to isolate an avirulence gene. By screening Pseudomonas strains that are known pathogens of crop plants related to Arabidopsis, highly virulent strains, P. syringae pv. maculicola (Psm) ES4326, P. syringae pv. tomato (Pst) DC3000, and an avirulent strain, Pst MM1065 were identified and analyzed as to their respective abilities to grow in wild type Arabidopsis thaliana plants (Dong et al., (1991) Plant Cell, 3:61-72; Whalen et al., (1991) Plant Cell 3:49-59; MM1065 is designated JL1065 in Whalen et al.). Psm ES4326 or Pst DC3000 can multiply 104 fold in Arabidopsis thaliana leaves and cause water-soaked lesions that appear over the course of two days. Pst MM1065 multiplies a maximum of 10 fold in Arabidopsis thaliana leaves and causes the appearance of a mildly chlorotic dry lesion after 48 hours. Thus, disease resistance is associated with severely inhibited growth of the pathogen.

Detailed Description Text (14):
The isolation of four Arabidopsis thaliana disease resistance mutants has been described using the cloned avrRpt2 gene to search for the host gene required for disease resistance to pathogens carrying the avrRpt2 gene (Yu et al., (1993), supra; Kunkel et al., (1993), supra). The four Arabidopsis thaliana mutants failed to develop an HR when infiltrated with Psm ES4326/avrRpt2 or Pst DC3000/avrRpt2 as expected for plants having lost their disease resistance capacity. In the case of one of these mutants, approximately 3000 five to six week old M.sub.2 ecotype Columbia (Col-0 plants) plants generated by ethyl methanesulfonic acid (EMS) mutagenesis were hand-inoculated with Psm ES4326/avrRpt2 and a single mutant, rps2-101C, was identified (resistance to Pseudomonas syringae) (Yu et al., (1993), supra).

Detailed Description Text (15):
The second mutant was isolated using a procedure that specifically enriches for mutants unable to mount an HR (Yu et al., (1993), supra). When 10-day old Arabidopsis thaliana seedlings growing on petri plates are infiltrated with Pseudomonas syringae pv. phaseolicola (Psp) NPS3121 versus Psp NPS3121/avrRpt2, about 90% of the plants infiltrated with Psp NPS3121 survive, whereas about 90%-95%

of the plants infiltrated with Psp NPS3121/avrRpt2 die. Apparently, vacuum infiltration of an entire small Arabidopsis thaliana seedling with Psp NPS3121/avrRpt2 elicits a systemic HR which usually kills the seedling. In contrast, contrast, seedlings infiltrated with Psp NPS3121 survive because Psp NPS3121 is a weak pathogen on Arabidopsis thaliana. The second disease resistance mutant was isolated by infiltrating 4000 EMS-mutagenized Columbia M.sub.2 seedlings with Psp NPS3121/avrRpt2. Two hundred survivors were obtained. These were transplanted to soil and re-screened by hand inoculation when the plants reached maturity of these 200 survivors, one plant failed to give an HR when hand-infiltrated with Psm ES4326/avrRpt2. This mutant was designated rps2-102C (Yu et al., (1993), surra).

Detailed Description Text (21):
The rps2 mutants displayed a HR when infiltrated with Pseudomonas pathogens carrying other avr genes, Psm ES4326/avrB, Pst DC3000/avrB, Psm ES4326/avrRpm1, Pst DC3000/avrRpm1. The ability to mount an HR to an avr gene other than avrRpt2 indicates that the rps2 mutants isolated by selection with avrRpt2 are specific to avrRpt2.

Detailed Description Text (74):
The invention will provide disease resistance to plants, especially crop plants, most especially important crop plants such as tomato, pepper, maize, wheat, rice and legumes such as soybean and bean, or any plant which is susceptible to pathogens carrying an avirulence gene, e.g., the avrRpt2 avirulence gene. Such pathogens include, but are not limited to, Pseudomonas syringae strains.

Other Reference Publication (17):
Kunkel et al.; RPS2, an Arabidopsis Disease Resistance Locus Specifying Recognition of Pseudomonas syringae Strains Expressing the Avirulence Gene avrRpt2; The Plant Cell, vol. 5, 865-875, Aug. 1993; Berkeley, CA.

Other Reference Publication (19):
Dong et al.; Induction of Arabidopsis Defense Genes by Virulent and Avirulent Pseudomonas syringae Strains and by a Cloned Avirulence Gene; The Plant Cell, vol. 3, 61-72, Jan. 1991; Columbus, Ohio.

Other Reference Publication (20):
Innes et al.; Molecular Analysis of Avirulence Gene avrRpt2 and Identification of a Putative Regulatory Sequence Common to All Known Pseudomonas syringae Avirulence Genes; J. Bacteriology vol. 175, No. 15. Aug. 1993, pp. 4859-4869; Berkeley, CA.

Other Reference Publication (23):
Whalen et al.; Identification of Pseudomonas syringae Pathogens of Arabidopsis and a a Bacterial Locus Determining Avirulence on Both Arabidopsis and Soybean; The Plant Cell. vol. 3, 49-59; Jan. 1991.

Other Reference Publication (24):
Guo-Liang Yu et al.; Arabidopsis Mutations at the RPS2 Locus Result in Loss of Resistance to Pseudomonas syringae Strains Expressing the Avirulence Gene avrRpt2; MPMI vol. 6, No. 4 pp. 434-443; Boston, MA, 1993.

Other Reference Publication (25):
Wanner et al.; Recognition of the Avirulence Gene avrB from Pseudomonas syringae pv. glycinea by Arabidopsis thaliana; MPMI, vol. 6, No. 5, 1993, pp. 582-591; Columbus.

Other Reference Publication (29):
Staskawicz et al.; Molecular Characterization of Cloned Avirulence Genes from Race 0 and Race 1 of Pseudomonas syringae pv. glycinea; Jour. of Bacterioloby, pp. 5789-5794, Dec. 1987.

Other Reference Publication (31):
Kobayashi et al.; Molecular Characterization of Avirulence Gene D from Pseudomonas syringae pv. tomato; Molecular Plant-Microbe Interactions, vol. 3, No. 2, pp. 94-102, 1990.

Other Reference Publication (32):
Kobayashi et al.; A Gene from Pseudomonas syringae pv. glycinea with Homology to Avirulence Gene D from P.s pv. tomatio but Devoid of the Avirulence Phenotype; MPM1 vol. 3, No. 2 pp. 103-111, 1990.

Other Reference Publication (33):
Arlat et al.; PopA1, a protein which induces a hypersensitivity-like response on specific Petunia genotypes, is secreted via the Hrp pathway of Pseudomonas solanacearum; The EMBO Jour. vol. 13 No. 3 pp. 543-553; 1994.

Other Reference Publication (38):
Midland et al.; The Structures of Syringolides 1 and 2, Novel C-Glycosidic Elicitors from Pseudomonas syringae pv. tomato; J. Org. Chem. vol. 58, pp. 2940-2945 2945 1993.

CLAIMS:

1. A substantially pure plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

9. The DNA of claim 1, wherein said DNA is operably linked to regulatory sequences for expression of said polypeptide, and wherein said regulatory sequences comprise a promoter.

14. The DNA of claim 13, wherein said DNA is operably linked to regulatory sequences for expression of said amino acid sequence, and wherein said regulatory sequences comprise a promoter.

21. The DNA of claim 18, wherein said DNA is operably linked to regulatory sequences for expression of said polypeptide, and wherein said regulatory sequences comprise a promoter.

25. A substantially pure plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1.

31. The DNA of claim 25, wherein said DNA is operably linked to regulatory sequences for expression of said polypeptide, and wherein said regulatory sequences comprise a promoter.

35. A substantially pure plant DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

41. The DNA of claim 35, wherein said DNA is operably linked to regulatory sequences for expression of said polypeptide, and wherein said regulatory sequences comprise a promoter.

45. A vector comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, said vector directing expression of the polypeptide

encoded by said DNA in a vector-containing cell.

48. A vector comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1, said vector directing expression of the polypeptide encoded by said DNA in a vector-containing cell.

49. A vector comprising plant DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, said vector directing expression of the polypeptide encoded by said DNA in a vector-containing cell.

50. A vector comprising DNA of the avrRpt2 gene (SEQ ID NO: 105) operably linked to plant regulatory sequences, wherein said plant regulatory sequences comprise a promoter.

51. A vector comprising (i) plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said potypeptide, resistance to a plant pathogen and (ii) DNA of the avrRpt2 gene (SEQ ID NO: 105), each of said DNAs operably linked to regulatory sequences which comprise a promoter.

52. A transformed bacterial cell comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

55. A transformed bacterial cell comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1.

56. A transformed bacterial cell comprising plant DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

57. A transformed plant cell comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

60. A transformed plant cell comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1.

61. A transformed plant cell comprising plant DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

64. The transformed plant cell of claim 62, said plant pathogen being Pseudomonas syringae.

66. A transformed plant cell of any one of claims 57-61, wherein said DNA is operably linked to regulatory sequences, and wherein said regulatory sequences

comprise a promoter.

71. A trangenic plant regenerated from a plant cell expressing (i) plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen and (ii) DNA of the avrRpt2 gene (SEQ ID NO: 105).

72. A transgenic plant comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen.

75. A transgenic plant comprising plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1, wherein said DNA is expressed in said transgenic plant.

76. A transgenic plant comprising plant DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said DNA is expressed in said transgenic plant.

79. A transgenic plant of any one of claims 71-76, wherein said DNA is operably linked to regulatory sequences for expression of said polypeptide, and wherein said regulatory sequences comprise a promoter.

85. A method of enhancing resistance to a plant pathogen in a plant, said method comprising:

(a) providing a transgenic plant cell that expresses plant DNA encoding a polypeptide comprising a P-loop and an LRR domain; and

(b) regenerating a transgenic plant from said plant cell wherein said DNA is expressed in said transgenic plant, and wherein said transgenic plant has enhanced resistance to a plant pathogen compared to a corresponding untransformed plant.

97. A method of enhancing resistance to a plant pathogen in a plant, said method comprising:

(a) providing a transgenic plant cell that expresses plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1; and

(b) regenerating a transgenic plant from said plant cell wherein said DNA is expressed in said transgenic plant, and wherein said transgenic plant has enhanced resistance to a plant pathogen compared to a corresponding untransformed plant.

101. A method of enhancing resistance to a plant pathogen in a plant, said method comprising:

(a) providing a transgenic plant cell that expresses plant DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain; and

(b) regenerating a transgenic plant from said plant cell wherein said DNA is expressed in said transgenic plant, and wherein said transgenic plant has enhanced resistance to a plant pathogen compared to a corresponding untransformed plant.

105. A method of enhancing resistance to a plant pathogen in a plant, said method comprising:

(a) providing a transgenic plant cell that expresses (i) plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring on a plant expressing said polypeptide, resistance to a plant pathogen, and (ii) DNA of the avrRpt2 gene (SEQ ID NO: 105); and

(b) regenerating a transgenic plant from said plant cell wherein each of said DNAs are expressed in said transgenic plant, and wherein said transgenic plant has enhanced resistance to a plant pathogen compared to a corresponding untransformed plant.

109. A method of producing a polypeptide comprising a P-loop and an LRR domain and which confers, on a plant expressing said polypeptide, resistance to a plant pathogen, said method comprising:

(a) providing a cell transformed with plant DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen;

(b) culturing said transformed cell to allow expression of said DNA; and

(c) isolating said polypeptide.

110. A method of producing a polypeptide comprising a P-loop and an LRR domain and which confers, on a plant expressing said polypeptide, resistance to a plant pathogen, said method comprising:

(a) providing a cell transformed with plant DNA encoding the amino acid sequence of SEQ ID NO: 2 from Met.sub.9 to Asn.sub.885 ;

(b) culturing said transformed cell to allow expression of said DNA; and

(c) isolating said polypeptide.

111. A method of producing a polypeptide comprising a P-loop and an LRR domain and which confers, on a plant expressing said polypeptide, resistance to a plant pathogen, said method comprising:

(a) providing a cell transformed with plant DNA encoding a plant resistance polypeptide having about 85% or greater sequence identity to SEQ ID NO: 1;

(b) culturing said transformed cell to allow expression of said DNA; and

(c) isolating said polypeptide.

112. A method of producing a polypeptide comprising a P-loop and an LRR domain and which confers, on a plant expressing said polypeptide, resistance to a plant pathogen, said method comprising:

(a) providing a cell transformed with DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen, wherein said P-loop and said LRR domain each comprise about 50% or greater sequence identity to a P-loop and an LRR domain of SEQ ID NO: 1;

(b) culturing said transformed cell to allow expression of said DNA; and

(c) isolating said polypeptide.

113. A method of producing a polypeptide comprising a P-loop and an LRR domain and which confers, on a plant expressing said polypeptide, resistance to a plant pathogen, said method comprising:

(a) providing a cell transformed with DNA having about 50% or greater sequence identity to SEQ ID NO: 1, said DNA encoding a polypeptide comprising a P-loop and an LRR domain, said polypeptide conferring, on a plant expressing said polypeptide, resistance to a plant pathogen;

(b) culturing said transformed cell to allow expression of said DNA; and

(c) isolating said polypeptide.

☐ 8. Document ID: US 6093556 A

L11: Entry 8 of 25 File: USPT Jul 25, 2000

DOCUMENT-IDENTIFIER: US 6093556 A
**** See image for Certificate of Correction ****
TITLE: Gene recombinant with biodegradability for chlorinated ethylene and bio-treatment of chlorinated ethylene therewith

Brief Summary Text (2):
The present invention relates to a gene recombinant with a recombinant DNA (in the context of this application this is often referred to only as "gene recombinant") capable of expressing a biodegradability for chlorinated ethylene under aerobic conditions and, in particular, to a gene recombinant of a bacteria of, such as, the genus Pseudomonas having this recombinant DNA as well as to a method for the biological treatment of chlorinated ethylene by such a recombinant.

Brief Summary Text (5):
A technique has been proposed in WO89/09827 for decomposing TCE, in which a toluene-monoxygenase gene originating from Pseudomonas mendocina KR-1 and having a biodegradability for TCE is introduced into a host bacterial cell by a foreign plasmid and the bacteria having this recombinant gene is used to effect biodegradation of TCE. However, this recombinant is obtained by introduction of a toluene-monoxygenase gene by a foreign plasmid and the toluene-monoxygenase gene is not carried on the chromosome, so that the efficiency of the biodegradation of trichloroethylene is low.

Brief Summary Text (6):
A technique of the biodegradation of TCE using a recombinant by recourse also to the use of a foreign plasmid is reported in the Journal of Fermentation and Bio-engineering, Vol. 79, No. 2, 100-106 (1995). This technique is based on the use of the phenol-hydroxylase gene of Pseudomonas putida BH. However, this recombinant also also exhibits a low capacity for decomposing chlorinated ethylene per unit cell population of as low as about 7.5 mg/liter.multidot.A.sub.600 (A.sub.600 is an absorbancy at 600 nm and serves as a parameter for the bacterial cell concentration). It is taught therein that the phenol-hydroxylase gene of Pseudomonas putida BH is composed of several components. It is also indicated in J. Ferment. and Bioeng., Vol. 79, No. 5, 485-488 (1995) that the phenol-hydroxylase gene of Pseudomonas putida BH is composed of several components.

Brief Summary Text (7):
In Japanese Patent Kokai Hei-6-105691 A, it is described that the phenol-hydroxylase (hereinafter sometimes abbreviated as PH) which is originated from the chromosomal DNA of the cell strain Pseudomonas putida KWI-9 decomposes trichloroethylene. Herein is described that a recombinant is prepared by introducing into the host cell a recombinant DNA having the PH gene and, downstream thereof, a terminator connected thereto, by a foreign plasmid. However, the capability of this recombinant for decomposing chlorinated ethylene is not sufficient, since the recombinant DNA is carried on the foreign plasmid.

Brief Summary Text (8):
Afterwards, it was confirmed by research at the gene level for the PH that the minimum unit of the PH gene for trichloroethylene-biodegradability consists of a group of chlorinated ethylene decomposing genes composed of 5 genes represented as pheA, pheB, pheC, pheD and pheE and that a gene for facilitating decomposition of chlorinated ethylene, which is denoted as pheZ, is present upstream from them. This bacteria Pseudomonas putida KWI-9 has the PH gene on the chromosomal DNA and exhibits a capability for biodegradation of chlorinated ethylene, though at a low level.

Brief Summary Text (9):
In Report of Environmental Engineering, Vol. 33, 165-175 (1996), a technique is given in which a tac-promotor is inserted upstream of the PH gene on the chromosome of the cell strain Pseudomonas putida KWI-9 to formulate a recombinant (Pseudomonas putida KN1-10A) which is used for biodegradation of trichloroethylene. However, this recombinant exhibits a low capacity for the biodegradation of chlorinated ethylene per unit cell population, since a promoter is inserted within the gene for facilitating decomposition of chlorinated ethylene, i.e. pheZ, and thus, the function of pheZ is obstructed.

Drawing Description Text (2):
FIG. 1 is a gene constructional diagram showing the organization of the phenol-hydroxylase gene of Pseudomonas putida KWI-9.

Detailed Description Text (5):
As the phenol-metabolizable bacteria having the biodegradability for chlorinated ethylene, there may be exemplified Pseudomonas putida KWI-9, Pseudomonas putida BH, Alkaligenes eutrophus JMP 134 (Applied and Environmental Microbiology, Vol. 62, No. 9, 3227-3233 (1996)) and others.

Detailed Description Text (6):
Among these phenol-metabolizable bacteria, preference is given for Pseudomonas putida KWI-9.

Detailed Description Text (7):
The PH gene existing in the chromosomal DNA of Pseudomonas putida KWI-9 is a gene group comprising a CE decomposing gene composed of 5 minimum units, pheA, pheB, pheC, pheD and pheE and, upstream thereof, a CE decomposition-facilitating gene pheZ. The gene arrangements for pheZ, pheA, pheB, pheC, pheD and pheE of Pseudomonas putida KWI-9 are given in SEQ ID NO: 1. The gene organizations of them are shown in FIG. 1.

Detailed Description Text (8):
The cell strain of Pseudomonas putida KWI-9 has been deposited at the Fermentation Research Institute Agency of Industrial Science and Technology with a Receipt No. FERM BP 6356 and the bacterial nature, method of culture thereof and so on are disclosed in Japanese Patent Kokai Hei-6-70753.

Detailed Description Text (11):

The recombinant DNA having inserted upstream of the PH gene group a promoter may preferably have, inserted downstream from the PH gene group, a terminator. When the chlorinated ethylene-decomposing gene group originates from Pseudomonas putida KWI-9, it is preferable that a terminator is inserted therein at a position downstream from the gene pheE.

Detailed Description Text (14):
The objective bacteria to be subjected to the gene recombination by inserting the recombinant DNA sequence into the chromosomal gene chain are not specifically restricted, while a bacterium of the genus Pseudomonas, in particular Pseudomonas putida KWI-9 may preferably be used therefor. When using Pseudomonas putida KWI-9, a first gene recombinant according to the present invention is obtained by inserting a promoter into the chromosomal DNA thereof at a position upstream of the PH gene group. For example, the following procedures may specifically be implemented.

Detailed Description Text (15):
A DNA fragment of a length of several thousands base pairs at a position before and after the initiation point of the PH gene group is first inserted into a plasmid vector having a conjugative ability. Then, the promoter can be inserted therein by making use of the cleavage site for a restriction enzyme existing upstream of the initiation point of the PH gene group. The so-prepared recombinant plasmid is then inserted into a bacterial cell of, for example, E. coli S17-1 strain, whereupon it is conjugated with Pseudomonas putida KWI-9 on a filter to effect a homologous recombination. Having been prepared in this manner, a gene recombinant having on its chromosome the above-mentioned recombinant DNA including the inserted promoter is selectively separated. When a DNA fragment having a capability for terminating gene transcription is to be inserted, a terminator can be inserted therein by making use of a restriction enzyme cleavage site existing downstream of the gene pheE, such as the portion of pheF, either before or after the insertion of the promoter.

Detailed Description Text (16):
Also in the case where the objective bacteria to be subjected to recombination of the gene are other than Pseudomonas putida KWI-9, a first gene recombinant according according to the present invention can be prepared in a similar way as above, so long as the objective bacteria have a PH gene group on their chromosomes.

Detailed Description Text (19):
The PH gene group in Pseudomonas putida KWI-9 can be isolated therefrom by a technique known per se using a restriction enzyme. A specific practice thereof is given in Japanese Patent Kokai Hei-6-105691 A in detail. Since the nucleotide sequence of the PH gene group is known as shown in SEQ ID NO: 1, it can be synthesized by Polymerase Chain Reaction (PCR) using the chromosome of Pseudomonas putida KWI-9 as the template.

Detailed Description Text (25):
As the phenol-metabolizable bacterium having a biodegradability for chlorinated ethylene, those which are used in the first gene recombinant according to the present invention given above may be used. A preferable one is Pseudomonas putida KWI-9, which is as described above for the first gene recombinant. When the phenol-hydroxylase gene originates from Pseudomonas putida KWI-9, it is preferable that a terminator is inserted downstream from the gene pheE.

Detailed Description Text (29):
When the chlorinated ethylene-decomposing gene originates from Pseudomonas putida KWI-9, it is preferable that a promoter is present at a position upstream from the CE decomposition-facilitating gene pheZ.

Detailed Description Text (31):

The objective bacteria to be subjected to the gene recombination by inserting the recombinant DNA sequence into the chromosomal gene chain are not specifically restricted, while a bacterium of the genus Pseudomonas, in particular Pseudomonas putida KWI-9 may preferably be used therefor. When using Pseudomonas putida KWI-9, a second gene recombinant according to the present invention is obtained by inserting a terminator into the chromosomal DNA thereof at a position downstream from the CE decomposing gene pheE. For example, the following procedures may specifically be implemented.

Detailed Description Text (32):
A DNA fragment of a length of several thousands base pairs at a position before and after the termination point of the CE decomposing gene pheE is first inserted into a plasmid vector having a conjugative ability. Then, a terminator can be inserted therein by making use of the cleavage site for a restriction enzyme existing downstream from the gene pheE, for example, at pheF. The so-prepared recombinant plasmid is then inserted into a bacterial cell of, for example, E. Coli S17-1 strain, whereupon it is conjugated with Pseudomonas putida KWI-9 on a filter to effect a homologous recombination. Having been prepared in this manner, a gene recombinant having on its chromosome the above-mentioned recombinant DNA including the inserted terminator is selectively separated.

Detailed Description Text (33):
In the case where the objective bacteria to be subjected to recombination of the gene are other than Pseudomonas putida KWI-9, a second gene recombinant according to to the present invention can be prepared in a similar way as above, so long as the objective bacteria have a CE decomposing gene on their chromosomes.

Detailed Description Text (41):
The culturing of the first or the second gene recombinant according to the present invention is carried out under a condition adapted for the growth of the gene recombinant. When the gene recombinant is Pseudomonas putida KWI-9, it is preferable to carry out the culturing using a carbon source and a nitrogen source, such as peptone, tripton and yeast extract, as well as inorganic salts, such as sodium chloride and potassium chloride, under an aerobic condition at a pH of 5-8.5, preferably 6-7, and a temperature of 15-35.degree. C., preferably around 30.degree. C.

Detailed Description Text (48):
Pseudomonas putida KWI-9

Detailed Description Text (57):
1. Preparation of Pseudomonas putida KN1-200A

Detailed Description Text (58):
The phenol-hydroxylase gene group exhibiting biodegradability for chlorinated ethylene coded on the chromosome of Pseudomonas putida KWI-9 is shown in FIG. 2 as a a gene map. The essential gene unit necessary for attaining biodegradation of chlorinated ethylene comprises five gene units pheA to pheE (CE decomposing gene group). The gene unit pheZ found upstream from pheA is the CE decomposition-facilitating gene. Upstream from pheZ, a tac-promotor was inserted by the procedures given below. Pseudomonas putida KN1-200A employed in Example 1 was prepared by a homologous recombination as given below (See FIG. 2).

Detailed Description Text (60):
Using the primer pair given below and using the chromosome of Pseudomonas putida KWI-9 as the template, a sequence of a length of 1.1 kb upstream of the gene pheZ was synthesized by PCR.

Detailed Description Text (67):
Using the primer pair given below and using Pseudomonas putida KWI-9 as the

template, pheZ and pheA were prepared by PCR.

Detailed Description Text (79):
The homologous part containing a tac-promotor was excised using PacI and was inserted into pMOK180 at its PacI site as shown in FIG. 2. The so-constructed plasmid was introduced into E. coli S17-1 cell strain and the resulting bacterium was caused to conjugate with Pseudomonas putida KWI-9 in such a manner as given in 7) below to effect a homologous recombination. By this, a gene recombinant Pseudomonas putida KN1-200A was obtained, in which the tac-promotor has been inserted in the chromosome upstream from the gene pheZ thereof, which was then separated selectively.

Detailed Description Text (81):
On carrying out the homologous recombination, the plasmid pMOK180 containing the tac-promotor, prepared as in 6) above, was introduced into a doner bacterium E. coli S17-1, whereupon it was cultured in an LB liquid culture medium (10 g of tripton, 5 g of yeast extract and 5 g of NaCl were dissolved in 1 liter of distilled water) overnight at 37.degree. C. Parallel therewith, a receptor bacterium of Pseudomonas putida KWI-9 strain was cultured in an LB medium at 30.degree. C. overnight. Each 0,5 ml of culture liquor was taken out from these cultures and the bacterial cells therein were collected on a centrifuge.

Detailed Description Text (83):
8) Selective Separation of Pseudomonas putida KN1-200A

Detailed Description Text (84):
The cell strain obtained in 7) above was cultured in an LB liquid culture medium overnight. A diluted culture liquor was spread on an LB agar medium containing 20 .mu.g/ml of XGal (namely, 5-bromo-5-chloro-3-indoyl-.beta.-D-galactoside) to develop colonies of the bacterium. White colonies found among the blue colonies were separated selectively as the cell strain in which pMOK180 had been deleted. Among them, a cell strain which turned yellow when sprayed with 0.1 M catechol (due to expression of pheF which codes catechol-2,3-oxygenase by the function of the tac-promotor) was selected as Pseudomonas putida KN1-200A which has a tac-promotor inserted upstream from pheZ.

Detailed Description Text (92):
Pseudomonas putida KN1-200A: The gene recombinant obtained as above, which was used in Example 1.

Detailed Description Text (93):
Pseudomonas putida KN1-10A: A gene recombinant in which a tac-promoter is held inserted into pheZ of Pseudomonas putida KWI-9, so as to cause the TCE biodegradation constitutively. In this gene recombinant, the function of pheZ is excluded due to the insertion of the tac-promoter into the gene pheZ. This gene recombinant was synthesized in accordance with the method reported in Environmental Engineering Search Reports, Vol. 33, 165-175 (1996) and was used in Comparative Example 1.

Detailed Description Text (99):
As seen in FIG. 4, while the biodegradabilities for the gene recombinant Pseudomonas putida KN1-200A (Example 1) in which a tac-promotor is held inserted upstream from pheZ of Pseudomonas putida KWI-9 and for the gene recombinant Pseudomonas putida KN1-10A (Comparative Example 1) in which the function of pheZ is destroyed, respectively, are almost the same up to about the 9.sup.th day, their biodegradabilities for TCE become different thereafter in such a manner that the TCE decomposition continues to proceed with Pseudomonas putida KN1-200A, whereas, for Pseudomonas putida KN1-10A, no progress of TCE decomposition is seen thereafter, thereafter, so that the TCE decomposition by the former amounts to 1.7 times that by the latter after 40 days.

Detailed Description Text (104):
Pseudomonas putida KN1-200A: The same as that in

Detailed Description Text (106):
Pseudomonas putida KN1 (pNIN205): This is a transformant synthesized by introducing a PH gene into the plasmid pRCT200 which have been integrated with a trc-promoter (this has the same function with tac-promoter) at the multicloning site thereof to obtain pNIN205, followed by introduction of this plasmid into Pseudomonas putida KWI-9. Details therefor are given below. This was used in Comparative Example 2.

Detailed Description Text (107):
2) Preparation of Pseudomonas putida KN1 (pNIN205)

Detailed Description Text (111):
The excised gene sequence from pheZ to pheE was inserted into pRCT200 at the portion from BamHI to XbaI of the multicloning site thereof. This recombinant plasmid was identified as pNIN205. At a position upstream from the multicloning site of pNIN205, there is integrated originally a trc-promotor, so that the inserted gene sequence starting from pheZ to pheE will be expressed constitutively. Downstream from the multicloning site of pNIN205, a terminator rrnBT.sub.1 T.sub.2 is integrated originally, the transcription of the gene will terminate at this position. This pNIN205 was introduced into Pseudomonas putida KN1 by electroporation to obtain the transformant Pseudomonas putida KN1 (pNIN205). The above-mentioned pRCT200 was synthesized in accordance with the method given in Example 3 in Japanese Patent Kokai Hei-6-105691.

Detailed Description Text (115):
The observed temporal change in the amount of consumption of TCE (given as a cumulative amount) by the biodegradation by Pseudomonas putida KN1-200A and by Pseudomonas putida KN1 (pNIN205), respectively, is shown in the graph of FIG. 5.

Detailed Description Text (116):
As seen from FIG. 5, the amount of consumption of TCE due to the biodegradation by Pseudomonas putida KN1 (pNIN205) (Comparative Example 2) became about one half of that by Pseudomonas putida KN1-200A (Example 2) after 40 days. From this, it is seen seen that the TCE-biodegradability of a gene recombinant having an integrated gene sequence containing a promoter, a TCE decomposition-facilitating gene and a TCE decomposing gene in the foreign plasmid per unit cell population is lower than that of a gene recombinant having such a gene sequence integrated in the chromosome.

Detailed Description Text (119):
1. Preparation of Pseudomonas putida KN1-210A

Detailed Description Text (120):
A terminator rrnBT.sub.1 T.sub.2 of 5SrRNA originating from E. coli was inserted into the chromosome of Pseudomonas putida KN1-200A prepared in Example 1, at its EcoT22I site including the initiation point (ATG) of the gene pheF. The practical procedures were as given below and performed in the same manner as in the case of the tac-promoter by means of a homologous recombination as explained in FIG. 6.

Detailed Description Text (128):
5) The homologous part and the inserted rrnBT.sub.1 T.sub.2 were excised by the PacI existing on both ends of pKNA82 and were inserted into pMOK170 at its PacI site. The so-obtained plasmid was introduced into cell strain E. coli S17-1, which was conjugated with Pseudomonas putida KN1-200A, whereby a gene recombinant Pseudomonas putida KN1-210A in which the terminator rrnBT.sub.1 T.sub.2 was integrated in EcoT22I of pheF on the chromosome by the homologous recombination was obtained and separated selectively. These procedures were performed in the same way as in the preparation and selection of the above-described Pseudomonas putida KN1-

200A.

Detailed Description Text (133):
As seen in FIG. 7, the gene recombinant of Example 3 brings about a high biodegradability for TCE all over the entire test period of 40 days which is even higher than that of the gene recombinant of Example 1. Thus, the gene recombinant Pseudomonas putida KN1-210A, in which a terminator is inserted into the gene sequence at a lower reach from the PH gene in the chromosome (Example 3), exhibits almost comparable biodegradability to that of the gene recombinant Pseudomonas putida KN1-200A (Example 1) for about four days from the start, but the TCE decomposing rate is higher thereafter for the gene recombinant having the integrated terminator. The amount of decomposition of TCE for Pseudomonas putida KN1-210A reached about 1.6 times that for Pseudomonas putida KN1-200A at the end of the 40 days' test period.

Detailed Description Text (138):
Pseudomonas putida KN1-210A: This is the same as that of Example 3.

Detailed Description Text (139):
Pseudomonas putida KN1 (pNIN205): This is the same as that of Comparative Example 2. 2.

Detailed Description Text (143):
The observed temporal change in the amount of decomposition of TCE by the biodegradation by Pseudomonas putida KN1 (pNIN205) (Comparative Example 3) and by Pseudomonas putida KN1-210A (Example 4), respectively, is shown in the graph of FIG. FIG. 8. The amount of decomposition of TCE by Pseudomonas putida KN1 (pNIN205) after a 40 days' test period was as low as 40 mg/liter which corresponds to about 3/10 of that by Pseudomonas putida KN1-210A. From this, it is seen that insertion of of a terminator downstream of the PH gene existing in a foreign plasmid gene does not cause an increase in the biodegradability of the resulting gene recombinant.

Detailed Description Paragraph Table (2):

SEQUENCE LISTING - - - - (1) GENERAL INFORMATION: - - (iii) NUMBER OF SEQUENCES: 8 - - - - (2) INFORMATION FOR SEQ ID NO: 1: - - (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 4800 base - #pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear - - (ii) MOLECULE TYPE: Genomic DNA - - (vi) ORIGINAL SOURCE: (A) ORGANISM: Pseudomonas - #putida (C) INDIVIDUAL ISOLATE: - #KWI-9 - - (ix) FEATURE: (A) NAME/KEY: peptide (B) LOCATION: 127..345 (C) IDENTIFICATION METHOD: - # E (D) OTHER INFORMATION: - #pheZ of phenol-hydroxylase - - (ix) FEATURE: (A) NAME/KEY: peptide (B) LOCATION: 434..1429 (C) IDENTIFICATION METHOD: - # E (D) OTHER INFORMATION: - #pheA of phenol-hydroxylase - - (ix) FEATURE: (A) NAME/KEY: peptide (B) LOCATION: 1440..1712 (C) IDENTIFICATION METHOD: - # E (D) OTHER INFORMATION: - #pheB of phenol-hydroxylase - - (ix) FEATURE: (A) NAME/KEY: peptide (B) LOCATION: 1754..3268 (C) IDENTIFICATION METHOD: - # E (D) OTHER INFORMATION: - #pheC of phenol-hydroxylase - - (ix) FEATURE: (A) NAME/KEY: peptide (B) LOCATION: 3301..3660 (C) IDENTIFICATION METHOD: - # E (D) OTHER INFORMATION: - #pheD of phenol-hydroxylase - - (ix) FEATURE: (A) NAME/KEY: peptide (B) LOCATION: 3689..4756 (C) IDENTIFICATION METHOD: - # E (D) OTHER INFORMATION: - #pheE of phenol-hydroxylase - - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: - #1: - - CCATTCCGGG TAAACGCGAA ATCCAGGGCG GCCAGAATCC CGACGCGCCT TG - #TGGCGCCG 60 - - TCGTTTGCGC CAGCTGGCAC GCTCTTCGCA ATAGAGGCCA TACCGCCCAT GC - #GAAGGAGC 120 - - AGCGCCATGA TCAACCAGTC GGTCCCCTTG TTTGAAGCCT CGCCCCGGTT CG - #TCCGCGTT 180 - - GATGGCCGCA CGCCCGAGGG CTTTGTGCAG TTCGCGTTCA GCGTGGCGGA CC - #CCGAGCTC 240 - - AATGTCGAGC TGATCATGCC CGAGCCGATG TTCGAGGCCT TCTGCAGCGT CA - #ACCACGTG 300 - - CGCTTCCTGC CGCCGGAGAT CCCCGCGCCC GATCAGGACG ACTGAGCTCG GC - #CAGTCCCC 360 - - ACGCACCGGA CCACGCACCC AACCACGCAC AGAACCACCC ACATAAAAAC AG - #CAAGACCC 420 - - AGGAGACCAA GCGATGCAAG TCGATATCAA GACCCAGCAG ATCCAGCCGC TG - #CGCCAGAC 480 - - CTACGGCCAT GTGGCGCGCC GCTTTGGCGA CAAGCCCGCC TCGCGCTACC AG - #GAGGCCAC 540 - - CTACGACGTG CAGTCGGAAG TGAATTTCCA

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CTACCGCCCG ACGTGGGACT CG - #CAACACGA 600 - - GATCTACGAC AAGCGCCGCA CCGCGATCGT
GATGGCGGAC TGGTACGCGC TC - #AAGGACCC 660 - - GCGCCAATAC TTCTACGGCG CTTACGTGAC
GGCGCGTGGC CGCCAGCAGG AT - #GCCACCGA 720 - - GAAGAGCTTC GCCTTCGTGG AAAAGCGCGG
CTTGCTGCAG GCGCTGCCTG CC - #GAATGGCA 780 - - GGACCGCCTG GCCGACGGCC TGCTGCCGCT
GCGCCATGTG GAGTGGGGCG CC - #AACATGAA 840 - - CAACTTCTAT TGCGCCGACT ACGGCTGGGG
CACGGCCATC ACCCAGGCCT GC - #ACCTACTG 900 - - CGCGATGGAT CGCCTCGGCA TTGCCCAGTA
CCTGTCGCGC ATCGGCATGC TG - #CTGGATGG 960 - - CAACACCGGT GTGGCGCTGG AGCGCGCCAA
GGTGGCCTGG ATGGAGAGCG CG - #GCGTGGCA 1020 - - GCCGATGCGC CGGCTGGTCG AGCGCAGCTT
TGTGATCGAG GACTGGTTCG AG - #ACCTTTGT 1080 - - CACCCAGAAC CTGGTGCTCG ACGGCCTGCT
TTACCCGCTG GTGTACCAGC AC - #GCCGACGC 1140 - - GGCCATCGTG CGCGCCTGCG GCACCGGGCT
GGCGGTGCTG ACCGAGTTCA TG - #AATGACTG 1200 - - GCGCGAGGAG CACGCACGCT GGGTCGACGC
GGTGATCCAG ACCGCCGCGG CA - #GAGTCCGA 1260 - - TGCCAATCGC ATGCTGCTGT CCGGCTGGGC
CCGCGCCGCG CGCGCGCAGG TG - #GCTGAGGC 1320 - - GCTGGTGCCG GTGGCCGACA CCTTGCTGGG
CGAGGGCGGC GAGCAGGTGG TG - #GCGCTGTG 1380 - - CGTGGAGCAG TTCGACGTGC GTCTTAACAA
GCTTGGCCTT GCCGCCTGAG GA - #AACCGCCA 1440 - - TGACCGCCAA TGTGTATATC GCGCTGCAGA
GCAACGACGA CACCCGTCCC AT - #CATCGAGG 1500 - - CCATCACCGA GGCCAACCCG CACGCGGTGG
TGTCGCAGTT TCCCGCCATG GT - #GAAGATCG 1560 - - ATGCGCCGGG ACACCTGACC ATCCTGCGCG
AACTGGTCTC CGACAAGCTC GG - #GCGCGACT 1620 - - GGGACCTGCA GGAGATTCAC CTGAACCTGA
TTTCGCTGTC GGGAAACATC GA - #CGAAGACG 1680 - - ACGACGCCTT CACCCTGCAC TGGAACGCCT
GAATCGGCCC GCCCACCGCA AA - #GACAAACG 1740 - - GAGACAAAAC ACCATGGACG CACGCAAGAA
GCTGAACCTG CGGGAAAAAT AC - #GCGACGAT 1800 - - GACGCGCGAC CTCGGCTGGG AAACCACCTA
CGAGCCGATG GACAAGGTCT TT - #CCCTACGA 1860 - - CAAGTACGAA GGCATCAAGA TCCATGACTG
GGACAAATGG GAAGACCCGT TT - #CGCATGAC 1920 - - CATGGACGCC TACTGGAAAT ACCAGTCGGA
GAAGGAGCGC AAGCTGTACG CG - #ATCATCGA 1980 - - TTCGTTCGTG CAGAACAATG GCCACCTCAA
CGTCTCGGAC CCGCGCTACC TG - #AACGCGCT 2040 - - GCGCCTGTTC CTGACCGGCG TGACGCCGCT
GGAGTACGCG GCGCACCGCG GC - #TATGCCCA 2100 - - CCTCGGGCGG CACTTTCGCG GCGCCGGCGC
GCGGGTGGCG GCGCAGATGC AG - #TCGATCGA 2160
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Other Reference Publication (1):
Takeo et al. Molecular cloning and sequencing of the phenol hydroxylase gene from pseudomonas putida BH. Journal of Fermentation and Bioengineering 79(5):485-488, 1995.

Other Reference Publication (6):
Applied and Environmental Microbiology, vol. 64, No. 1, (Jan. 1998) Yee et al, Rhizoremediation of Trichloroethylene by a Recombinant, Root-Colonizing Pseudomonas fluorescens. . . , pp. 112-118.

Other Reference Publication (7):
Applied and Environmental Microbiology, vol. 64, No. 7 (Jul. 1998) Berendes et al, Construction and Use of an ipb DNA Module to Generate Pseudomonas Strains. . . , pp. 2454-2462.

CLAIMS:

1. A gene recombinant capable of expressing a biodegradability for chlorinated ethylene, said gene recombinant comprising a recombinant DNA sequence carried on a chromosome, said recombinant DNA sequence comprising:

a phenol-hydroxylase gene originating from a phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene, said phenol-hydroxylase gene comprising a gene for facilitating decomposition of chlorinated ethylene and a series of genes for decomposing chlorinated ethylene; and

a promoter inserted upstream of the gene for facilitating decomposition of chlorinated ethylene by 1-500 base pairs.

2. A gene recombinant capable of expressing a biodegradability for chlorinated ethylene, said gene recombinant comprising a recombinant DNA sequence carried on a chromosome, said recombinant DNA sequence comprising:

a phenol-hydroxylase gene originating from a phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene, said phenol-hydroxylase gene comprising a gene for facilitating decomposition of chlorinated ethylene and a series of genes for decomposing chlorinated ethylene; and

a terminator inserted downstream of the phenol-hydroxylase gene by 1-500 base pairs. pairs.

3. A gene recombinant capable of expressing a biodegradability for chlorinated ethylene, said gene recombinant comprising a recombinant DNA sequence carried on a chromosome, said recombinant DNA sequence comprising:

a phenol-hydroxylase gene originating from a phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene, said phenol-hydroxylase gene comprising a gene for facilitating decomposition of chlorinated ethylene and a series of genes for decomposing chlorinated ethylene;

a promoter inserted upstream of the gene for facilitating decomposition of chlorinated ethylene by 1-500 base pairs; and

a terminator inserted downstream of the phenol-hydroxylase gene by 1-500 base pairs.

4. A gene recombinant capable of expressing a biodegradability for chlorinated ethylene, said gene recombinant comprising a recombinant DNA sequence carried on a chromosome, said recombinant DNA sequence comprising:

a phenol-hydroxylase gene originating from Pseudomonas putida KWI-9, said phenol-hydroxylase gene comprising a gene for facilitating decomposition of chlorinated ethylene comprising pheZ and a series of genes for decomposing chlorinated ethylene comprising pheA, pheB, pheC, pheD and pheE; and

a promoter inserted upstream of the gene for facilitating decomposition of chlorinated ethylene by 1-500 base pairs.

5. A gene recombinant capable of expressing a biodegradability for chlorinated ethylene, said gene recombinant comprising a recombinant DNA sequence carried on a chromosome, said recombinant DNA sequence comprising:

a phenol-hydroxylase gene originating from Pseudomonas putida KWI-9, said phenol-hydroxylase gene comprising a gene for facilitating decomposition of chlorinated ethylene comprising pheZ and a series of genes for decomposing chlorinated ethylene comprising pheA, pheB, pheC, pheD and pheE; and

a terminator inserted downstream of the phenol-hydroxylase gene by 1-500 base pairs.

6. A gene recombinant capable of expressing a biodegradability for chlorinated ethylene, said gene recombinant comprising a recombinant DNA sequence carried on a chromosome, said recombinant DNA sequence comprising:

a phenol-hydroxylase gene originating from Pseudomonas putida KWI-9, said phenol-hydroxylase gene comprising a gene for facilitating decomposition of chlorinated ethylene comprising pheZ and a series of genes for decomposing chlorinated ethylene comprising pheA, pheB, pheC, pheD and pheE;

a promoter inserted upstream of the gene for facilitating decomposition of chlorinated ethylene by 1-500 base pairs; and

a terminator inserted downstream of the phenol-hydroxylase gene by 1-500 base pairs. pairs.

7. The gene recombinant of claim 1, wherein said recombinant DNA sequence comprises SEQ ID NO: 1.

8. The gene recombinant of claim 2, wherein said recombinant DNA sequence comprises SEQ ID NO: 1.

9. The gene recombinant of claim 3, wherein said recombinant DNA sequence comprises SEQ ID NO: 1.

10. The gene recombinant of claim 4, wherein said recombinant DNA sequence comprises comprises SEQ ID NO: 1.

11. The gene recombinant of claim 5, wherein said recombinant DNA sequence comprises comprises SEQ ID NO: 1.

12. The gene recombinant of claim 6, wherein said recombinant DNA sequence comprises comprises SEQ ID NO: 1.

13. A gene recombinant as claimed in claim 1, wherein the phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene belongs to the genus Pseudomonas.

14. A gene recombinant as claimed in claim 1, wherein the phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene is Pseudomonas putida KWI-9.

15. A gene recombinant as claimed in claim 1, wherein the phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene is Pseudomonas putida KWI-9 and the gene recombinant comprises the recombinant DNA sequence carried on the chromosome of Pseudomonas putida KWI-9.

16. A gene recombinant as claimed in claim 1, wherein the phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene is Pseudomonas putida KWI-9 and the phenol-hydroxylase gene comprises a gene for facilitating decomposition of chlorinated ethylene comprising pheZ and a series of genes for decomposing chlorinated ethylene comprising pheA, pheB, pheC, pheD and pheE.

17. A gene recombinant as claimed in claim 1, wherein the phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene is Pseudomonas putida KWI-9, and the phenol-hydroxylase gene comprises a gene facilitating decomposition of chlorinated ethylene comprising pheZ, a series of genes for decomposing chlorinated ethylene comprising pheA, pheB, pheC, pheD and pheE and the gene recombinant comprises the recombinant DNA sequence carried on the chromosome of Pseudomonas putida KWI-9.

18. A gene recombinant as claimed in claim 2, wherein the terminator has a stem-and and-loop structure.

19. A gene recombinant as claimed in claim 4, wherein the phenol-metabolizable bacterium capable of bio-degrading chlorinated ethylene is Pseudomonas putida KWI-9 and the gene recombinant comprises the recombinant DNA sequence carried on the chromosome of Pseudomonas putida KWI-9.

20. A method of biological treatment of chlorinated ethylene comprising

subjecting chlorinated ethylene to a biological digestion by a gene recombinant as defined in claim 17.

21. A method of biological treatment of chlorinated ethylene comprising

subjecting chlorinated ethylene to a biological digestion by a gene recombinant as defined in claim 19.

22. A method of biological treatment of chlorinated ethylene comprising

subjecting chlorinated ethylene to a biological digestion by a gene recombinant as defined in claim 19.

☐ 9. Document ID: US 6033874 A

L11: Entry 9 of 25 File: USPT Mar 7, 2000

DOCUMENT-IDENTIFIER: US 6033874 A
TITLE: CRY1C polypeptides having improved toxicity to lepidopteran insects

Brief Summary Text (37):
A further aspect of the invention is a host cell which comprises one or more of the nucleic acid segment disclosed herein which encode a modified Cry1* protein. Preferred host cells include bacterial cells, such as E. coli, B. thuringiensis, B. subtilis, B. megaterium, or Pseudomonas spp. cells, with B. thuringiensis NRRL B-21590, NRRL B-21591, NRRL B-21592, NRRL B-21638, NRRL B-21639, NRRL B-21640, NRRL B-21609, and NRRL B-21610 cells being highly preferred. Another preferred host cell is an eukaryotic cell such as a fungal, animal, or plant cell, with plant cells such as grain, tree, vegetable, fruit, berry, nut, grass, cactus, succulent, and ornamental plant cells being highly preferred. Transgenic plant cells such as corn, rice, tobacco, potato, tomato, flax, canola, sunflower, cotton, wheat, oat, barley, and rye cells are particularly preferred.

Brief Summary Text (177):
In one preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension comprising lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferably the cells are B. thuringiensis cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein is contemplated to be useful, such as Bacillus spp., including B. megaterium, B. subtilis; B. cereus, Escherichia spp., including E. coli, and/or Pseudomonas spp., including P. cepacia, P. aeruginosa, and P. fluorescens. Alternatively, the oil flowable suspension may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (178):
In a second preferred embodiment, the bioinsecticide composition comprises a water dispersible granule or powder. This granule or powder may comprise lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferred sources for these compositions include bacterial cells such as B. thuringiensis cells, however, bacteria of the genera Bacillus, Escherichia, and Pseudomonas which have been transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Alternatively, the granule or powder may consist of a combination of

one or more of the following compositions: lysed or unlysed bacterial cells, spores, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (179):
In a third important embodiment, the bioinsecticide composition comprises a wettable powder, spray, emulsion, colloid, aqueous or organic solution, dust, pellet, or collodial concentrate. Such a composition may contain either unlysed or lysed bacterial cells, spores, crystals, or cell extracts as described above, which contain one or more of the novel crystal proteins disclosed herein. Preferred bacterial cells are B. thuringiensis cells, however, bacteria such as B. megaterium, B. subtilis, B. cereus, E. coli, or Pseudomonas spp. cells transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or alternatively, dissolve in a controlled-release, sustained-release, or other time-dependent manner. Alternatively, such a composition may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Detailed Description Text (16):
The nucleotide sequences of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the sites of lepidopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B. thuringiensis toxin.

Detailed Description Text (17):
Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility or toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and Gram-positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium; Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae, Actinomycetales, and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

Detailed Description Text (19):
Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp., phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Streptomyces lividans and the like.

Detailed Description Text (22):
A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera Bacillus, Pseudomonas, Erwinia, Serratia, Klebsiella, Zanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes; fungi, particularly yeast, e.g., genera Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phytosphere bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodobacter sphaeroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes eutrophus, and Azotobacter vinlandii; and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans.

Detailed Description Text (54):
In an alternate embodiment, the recombinant expression of DNAs encoding the crystal proteins of the present invention is performed using a transformed Gram-negative bacterium such as an E. coli or Pseudomonas spp. host cell. Promoters which function function in high-level expression of target polypeptides in E. coli and other Gram-negative host cells are also well-known in the art.

CLAIMS:

1. An isolated Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide comprising one or more amino acid mutations in the loop region between .alpha. helices 6 and 7 of domain 1, wherein the polypeptide has improved insecticidal activity against Lepidopteran insects relative to a native Cry1.delta.-endotoxin polypeptide.

7. A composition comprising a Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide, wherein the polypeptide comprises one or more amino acid mutations in the loop region between .alpha. helices 6 and 7 of domain 1, and the polypeptide has has improved insecticidal activity against Lepidopteran insects relative to a native Cry1C .delta.-endotoxin polypeptide.

17. An isolated nucleic acid segment encoding a Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide, wherein the polypeptide comprises one or more amino acid mutations in the loop region between .alpha. helices 6 and 7 of domain 1, 1, and the polypeptide has improved insecticidal activity against Lepidopteran insects relative to a native Cry1C .delta.-endotoxin polypeptide.

21. A nucleic acid vector comprising operably linked in the 5' to 3' direction:

a promoter which directs transcription of a nucleic acid segment;

a nucleic acid segment encoding a Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide wherein:

the polypeptide comprises one or more amino acid mutations in the loop region between .alpha. helices 6 and 7 of domain 1, and

the polypeptide has improved insecticidal activity against Lepidopteran insects relative to a native Cry1C .delta.-endotoxin polypeptide;

a 3' transcriptional termination signal; and

a 3' polyadenylation signal.

23. The nucleic acid vector of claim 22, wherein the nucleic acid segment encodes an an alanine residue for amino acid Lys219 in the loop region of the polypeptide.

29. The nucleic acid vector of claim 28, wherein the plant-expressible promoter is selected from the group consisting of corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, pea small subunit RuBP carboxylase, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, Potato patatin, lectin, CaMV 35S, and S-E9 small subunit RuBP carboxylase promoters.

30. A host cell comprising operably linked in the 5' to 3' direction:

a promoter which directs transcription of a nucleic acid segment;

a nucleic acid segment encoding a Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide wherein:

the polypeptide comprises one or more amino acid mutations in the loop region between .alpha. helices 6 and 7 of domain 1, and

the polypeptide has improved insecticidal activity against Lepidopteran insects relative to a native Cry1C .delta.-endotoxin polypeptide;

a 3' transcriptional termination signal; and

a 3' polyadenylation signal.

32. The host cell of claim 30, wherein the host cell is an E. coli, Bacillus thuringiensis, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, or Pseudomonas spp. cell.

37. A transgenic plant comprising operably linked in the 5' to 3' direction:

a promoter which directs transcription of a nucleic acid segment;

a nucleic acid segment encoding a Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide wherein:

the polypeptide comprises one or more amino acid mutations in the loop region between .alpha. helices 6 and 7 of domain 1, and

the polypeptide has improved insecticidal activity against Lepidopteran insects relative to a native Cry1C .delta.-endotoxin polypeptide;

a 3' transcriptional termination signal; and

a 3' polyadenylation signal.

47. A method of controlling Lepidopteran insects comprising contacting said insects with an insecticidally-effective amount of a Bacillus thuringienis Cry1C .delta.-endotoxin polypeptide, wherein:

the polypeptide comprises at least one amino acid mutation in the loop region between .alpha. helices 6 and 7 of domain 1; and

the polypeptide has improved insecticidal activity against Lepidopteran insects relative to a native Cry1C .delta.-endotoxin polypeptide.

☐ 10. Document ID: US 6022950 A

L11: Entry 10 of 25 File: USPT Feb 8, 2000

DOCUMENT-IDENTIFIER: US 6022950 A
**** See image for Certificate of Correction ****
TITLE: Hybrid molecules having translocation region and cell-binding region

Abstract Text (4):
(c) wherein said third part comprises a chemical entity to be introduced into the cell, wherein each of said first part and said third part is non-native with respect to said naturally occurring protein, and further wherein said covalent bond connecting said second part and said third part is a cleavable bond, provided that when said second part comprises a portion of a translocation domain of Pseudomonas exotoxin, said third part is not a polypeptide.

Brief Summary Text (7):
The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps: (i) the binding domain of diphtheria toxin binds to specific receptors on the surface of a sensitive cell; (ii) while bound to its receptor, the toxin molecule is internalized into an endocytic vesicle; (iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage in 1.sub.1 between fragments A and B; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin spontaneously inserts into the endosomal membrane; (v) once embedded in the membrane, the translocation domain of the toxin facilitates the delivery of Fragment A into the cytosol; (vi) the catalytic activity of Fragment A (i.e., the nicotinamide adenine dinucleotide-dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the intoxicated cell. It is apparent that a single molecule of Fragment A introduced into the cytosol is sufficient to shut down the cell's protein synthesis machinery and kill the cell. The mechanism of cell killing by Pseudomonas exotoxin A, and possibly by certain other naturally-occurring toxins, is is very similar.

Brief Summary Text (11):
(b) the second part including a portion of a translocation domain of a protein, provided that (i) the hybrid molecule does not include an enzymatically-active portion of such protein, (ii) the first part and the second part are not segments of the same naturally-occurring polypeptide toxin, and (iii) the portion of the translocation domain, when covalently bonded to an enzymatically-active portion (i.e., the "effector region") of a toxin selected from diphtheria toxin, Pseudomonas exotoxin A, cholera toxin, ricin toxin, and Shiga-like toxin, is capable capable of translocating such effector region across the cytoplasmic membrane of the cell. "Translocation" here means the facilitation of movement of a chemical entity from the exterior surface of a cellular membrane (or what constituted the exterior surface prior to formation of an endocytic vesicle), through the membrane, and into the cytosol at the interior of the cell. A "translocation domain" is a segment of a protein which, when the protein is bound to the exterior surface of a cellular membrane, is capable of translocating some portion of that protein through the membrane.

Brief Summary Text (16):
In preferred embodiments, the second part comprises at least a portion of the translocation domain of a naturally-occurring toxin (e.g. diphtheria toxin or Pseudomonas exotoxin A), and the ligand comprises a hormone (e.g. a polypeptide hormone such as insulin, Interleukin II (also termed "IL2"), Interleukin IV, Interleukin VI or EGF, or, alternatively, a steroid hormone); an antigen-binding, single-chain analog of a monoclonal antibody; or a polypeptide toxin capable of binding to the desired class of cells (more preferably, both the first and the second parts are derived from diphtheria toxin); where both the first and second parts are polypeptides, the hybrid molecule is preferably a recombinant protein; the hybrid molecule preferably additionally comprises a third part which is connected to the second part by at least one covalent bond and which is a chemical entity to be introduced into the cell (provided that where the third part is a polypeptide, the cleavable bond is a disulfide bond). More preferably, all three parts are polypeptides and the hybrid molecule is a recombinant protein (that is, a protein produced by recombinant DNA techniques); the third part and the second part are linked through a proteolytically-sensitive disulfide loop (defined below); the third part is an antigen-binding, single-chain analog of a monoclonal antibody (where such antigen is, for example, a viral protein such as the human immunodeficiency virus (HIV) protease), or alternatively, the enzymatically active portion of an enzyme (e.g., hexosaminidase A; .alpha.-1,4-glucosidase; phenylalanine hydroxylase; a protease; a nuclease; or a toxin such as cholera toxin, LT toxin, C3 toxin, Shiga toxin, E.coli Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin or Pseudomonas exotoxin A), and most preferably it supplies an enzymatic activity in which the cell is deficient, as, for example, in the case of a genetic deficiency. Where the enzyme is cholera toxin, the resulting hybrid molecule may be used to raise the cyclic AMP level within an animal cell: preferably, the cell so treated is a T-cell and the hybrid molecule includes at least a portion of the binding domain of IL2. By "proteolytically-sensitive disulfide loop" is meant a sequence of at least 5 amino acid residues (preferably from 6 to 30, and more preferably from 11 to 18) joined in series by peptide bonds, the first and last residues of which sequence are Cys residues which link to form a cystine disulfide bond. At least two of the remaining residues of the sequence together create a proteolytically-sensitive site: i.e., a peptide bond formed between two residues, the second (carboxyl side) of which may be, e.g., Arg, Lys, Phe, Tyr, or Trp. There is preferably also at least one Ser residue within the sequence of the loop. The loop, which may be a naturally-occurring feature of the second part or the third part, or may be engineered (e.g., from a synthetic DNA sequence) into the hybrid, joins the third part to the second part by two types of covalent linkages, peptide and sulfhydryl, ensuring that these two portions of the hybrid will remain associated with each other, even in the presence of extracellular proteases, until after the hybrid has bound to the target cell, but will separate at the appropriate stage. Both the proteolytically-sensitive peptide bond(s) within the disulfide loop and the disulfide bond itself are cleaved at some point prior to or during passage of the chemical entity through the cellular membrane of the endocytic vesicle, resulting in the release of the chemical entity into the cytosol, free of the receptor-bound cell-binding ligand portion (the first part), and translocation domain portion (the second part) of the hybrid.

Brief Summary Text (22):
Based upon the observation that certain types of polypeptide toxins have three separate functional regions, one region which binds the molecule to particular receptors on the surface of a target cell, a second one which facilitates entry of the enzymatically-active region into the cytosol of the cell, and a third region which exhibits the enzymatic activity that characterizes the toxic effect of the molecule, the invention comprises bi- or tripartite hybrid molecules in which any of these regions may be replaced with functionally comparable regions from other sources. That is, the first functional region may be replaced with a particular binding moiety which binds the hybrid molecule to a selected class of cells, such

as IL2 (which binds to high-affinity IL2 receptor-bearing T-cells), or .alpha. melanocyte stimulating hormone (.alpha.MSH, which binds to melanocytes), or a moiety moiety which binds to a broad spectrum of cell types, as is characteristic of the binding domains of cholera toxin and diphtheria toxin; the second part may be taken from any type of polypeptide in which a translocation domain is identifiable, but will most likely be from a toxin molecule that translocates in a manner similar to diphtheria toxin and Pseudomonas exotoxin A. The optional third part may be any type type of moiety that one wants to insert into the cell and that will fit through the channel in the membrane formed by the translocation domain: for example, a cell-killing enzyme such as Shiga toxin; a metabolic enzyme such as phenylalanine hydroxylase (the enzyme in which phenylketonurics are deficient); an antigen-binding, single-chain analog of a monoclonal antibody against an antigen that appears within the target cell; or a fluorescent label.

Detailed Description Text (3):
Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin and Pseudomonas exotoxin A, and may include other toxins and non-toxin molecules, as well. The translocation domains of diphtheria toxin and Pseudomonas exotoxin A are well characterized (see, e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82:1692-1696, 1985; Colombatti et al., J. Biol. Chem. 261:3030-3035, 1986; and Deleers et al., FEBS 160:82-86, 1983), and the existence and location of such a domain in other molecules may be determined by methods such as those employed by Hwang et al., Cell 48:129-136, 1987; and Gray et al., Proc. Natl. Acad. Sci. USA 81:2645-2649, 1984.

Detailed Description Text (57):
The translocation function of the hybrid molecule may be supplied by an appropriate piece of a polypeptide other than diphtheria toxin, but which is capable of translocating in a manner analogous to that of diphtheria toxin (e.g., Pseudomonas exotoxin A, botulinum neurotoxin, or ricin), or in any other manner which accomplishes the objective of translocating the functional "third part" of the hybrid molecule into the cell's cytoplasm.

Detailed Description Text (60):
Likewise, the hybrid of the invention will be useful for specifically destroying certain cells. Besides the cholera toxin A.sub.1 -hybrid, ricin A-hybrid and Shiga-like toxin A-hybrid exemplified above, a cell-killing function may be provided by the enzymatically-active portion of any polypeptide toxin, including but not limited to LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin and Pseudomonas exotoxin A. Cells to be targeted might include cancer cells, virus-infected cells, or adipocytes.

CLAIMS:

1. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding ligand effective to cause said hybrid molecule to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a chemical entity to be introduced into the cell, wherein each of said first part and said third part is non-native with respect to said naturally occurring protein, and further wherein said covalent bond connecting said second part and said third part is a cleavable bond, provided that when said second part comprises a portion of a translocation domain of Pseudomonas

exotoxin, said third part is not a polypeptide.

5. The hybrid molecule of claim 1, wherein the covalent bond connecting said second and third parts is a disulfide bond.

21. The hybrid molecule of claim 4, wherein the covalent bond connecting said second and third parts is a disulfide bond.

22. The hybrid molecule of claim 4, wherein the covalent bond connecting said second and third parts is a peptide bond or a proteolytically-sensitive disulfide loop.

26. The hybrid molecule of claim 20, wherein said toxin is selected from the group consisting of cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin, and Pseudomonas exotoxin A.

33. The hybrid molecule of claim 27, which is a recombinant protein.

42. The hybrid molecule of claim 32, wherein the covalent bond connecting said second and third parts is a disulfide bond.

43. The hybrid molecule of claim 34, wherein said third part is a polypeptide and the covalent bond connecting said second and third parts is a peptide bond or a proteolyticaily-sensitive disulfide loop.

45. The hybrid molecule of claim 44, which is a recombinant hybrid protein.

51. The hybrid molecule of claim 49, wherein said covalent bond comprises a proteolytically sensitive disulfide loop.

☐ 11. Document ID: US 5998191 A

L11: Entry 11 of 25 File: USPT Dec 7, 1999

DOCUMENT-IDENTIFIER: US 5998191 A
**** See image for Certificate of Correction ****
TITLE: High specificity homocysteine assays for biological samples

Brief Summary Text (12):
An additional procedure for detection of homocysteine that involves use of a microbial enzyme from the bacterium Pseudomonas ovalis is disclosed in Yamaguchi et al., Annual Report of Sapporo City Institute of Public Health, No. 20, pp. 67-74, 1993. The authors focused on detection of the amino acid methionine as an indirect method to determine homocysteine levels (such as for homocystinuria patients), since homocysteine was considered unstable, for example being prone to disulfide bonding with proteins.

Brief Summary Text (47):
(a) a homocysteinase from Trichomonas vaginalis, or Pseudomonas (such as species ovalis or putida), and

Brief Summary Text (49):

In a further aspect of the invention, there is provided a chimeric nucleotide sequence (whether of DNA or RNA), derived from more than one gene, or other polynucleotide, that codes for a homocysteinase, wherein expression of such sequence sequence leads to the production of a chimeric homocysteinase. Such homocysteinases have valuable properties with respect to the practice of the invention, and a preferred example thereof includes a chimeric enzyme that comprises amino acid sequences derived from both Trichomonas vaginalis and Pseudomonas putida homocysteinases.

Brief Summary Text (53):
Pseudomonas, Clostridium, Aeromonas or Trichomonas, and in an example thereof, one or more peptide sequences of such an enzyme are correspondingly replaced by one or more homologous peptide sequences of SEQ ID NO:10, for example, those selected from the group consisting of:

Detailed Description Text (9):
A first homocysteinase that is preferred in the practice of the invention is L-methionine-alpha-deamino-gamma-mercaptomethane lyase (methionine lyase) derived from the bacterial source, Pseudomonas putida. The enzyme has been purified by S. Ito et al., Journal of Biochemistry, 79, pp.1263-1272 (1976), and determined to have a molecular weight of about 170kDa. In the context studied by S. Ito, the enzyme carried out the alpha-gamma elimination of methionine to alpha-keto butyrate, methanethiol, and ammonia. If homocysteine is the substrate, then alpha-keto butyrate, hydrogen sulfide, and ammonia would be the resultant products. The homologous enzyme has been isolated from Pseudomonas ovalis, H. Tanaka et al., Biochemistry ,16, pp.100-106 (1977). Methods for the recombinant production of this Pseudomonas enzyme have also been developed (see Y. Tan et al., Protein Expression and Purification, 9, pp. 233-245, 1997), and use of recombinant enzyme in the clinical practice of the present invention is expected to provide advantages in terms of diagnostic kit cost and assay reproducibility. The Tan et al. reference describes construction of a clone designated pAC1-1 containg a single copy of enzyme-encoding sequence. An additional clone, designated pAC1-1, has been constructed which contains two copies of the encoding sequence, in tandem. As with the pAC1-1 structure, the pT7-7 plasmid was used to construct pAC1-11. The two encoding sequences are linked together at a BamHI site, with the first gene linked NdeI to BamHI, and the second linked BamHI to a further BamHI site.

Detailed Description Text (10):
The substrate specificity of the P. putida enzyme has also been determined. For example, N. Esaki et al. Methods in Enzymology, 143, pp. 459-465 (1987) report that on a relative activity scale where activity toward methionine is assigned 100, cysteine is 10, and homocysteine is 180. That the enzyme is reactive to all three sulfhydryl-containing amino acids underscores the need to define clinical assays for which the source of hydrogen sulfide can be properly determined. It should be noted that the apparent 10- fold preference of the enzyme for homocysteine over cysteine does not take into account that the concentration of cysteine in a biological sample may be high--in fact much higher than the concentration of homocysteine. Homocysteinase enzymes of suitable catalytic activity can be derived from other Pseudomonas species, or from other bacteria, using routine screening procedures and assays that are recognized in the art.

Detailed Description Text (14):
Use of a recombinant version of the T. vaginalis enzyme is also preferred. One potential cloning strategy follows the observations by A. Marcos et al., FEMS Microbiology Letters, 135, pp. 259-264 (1996), that T. vaginalis genes may have few introns. Accordingly, a genomic library would be constructed (see D. E. Riley et al. Molecular and Biochemical Parasitology, 51, pp. 161-164, 1992) and screened with DNA fragments corresponding to the Psuedomonas putida enzyme, and which are expected to reflect some partially conserved sequence. Lockwood et al. also list other reports of bacteria having methionine-gamma-lyase activity involving species

of Pseudomonas, Clostridium, and Aeromonas.

Detailed Description Text (20):
Such homocysteinases have properties that are very useful with respect to the practice of the invention, and a preferred example thereof includes a chimeric enzyme that comprises amino acid sequences corresponding to both Trichomonas vaginalis and Pseudomonas putida homocysteinases.

Detailed Description Text (25):
The present invention therefore provides for a purified and isolated DNA molecule comprising a chimeric nucleotide sequence that encodes amino acid sequence of Pseudomonas putida homocysteinase, and amino acid sequence of Trichomonas vaginalis homocysteinase (derived from either mgl1, or mgl2, or both) from which can be expressed a functional protein having homocysteinase activity. In a preferred aspect, the nucleotide construct (or corresponding amino acid construct) corresponds predominantly to that of P. putida, and thus there is provided a DNA molecule that comprises an encoding nucleotide sequence for Pseudomonas putida homocysteinase, wherein one or more subsequences thereof that encode one or more amino acids of said enzyme are correspondingly replaced by one or more nucleotide subsequences that encode the corresponding amino acids of a Trichomonas vaginalis homocysteinase. In the practice of the present invention, the T. vaginalis enzyme encoded by the mgl1 gene is hereinafter referred to as the T1 protein, and that encoded by the mgl2 gene is referred to as the T2 protein.

Detailed Description Text (29):
The invention therefore provides for a DNA molecule that comprises an encoding sequence for Pseudomonas putida homocysteinase, wherein one or more subsequences thereof, that each encode one or more amino acids of said enzyme, are correspondingly replaced by one or more nucleotide subsequences than each encode the one or more corresponding amino acids of a Trichomonas vaginalis homocysteinase, and wherein said resultant amino acid replacements are selected from the group consisiting of:

Detailed Description Text (30):
(a) Cys-Ser-Arg-Ala-Asp-Ile-Ile-Ala-Lys-Val-Lys-Ser (SEQ ID NO: 1), or any subset thereof, from Trichomonas vaginalis (T2) for Val-Gly-Ser-Gln-Ala-Leu-Val-Asp-Arg-Ile-Arg-Leu (SEQ ID NO: 2), or any subset thereof, from Pseudomonas putida;

Detailed Description Text (31):
(b) Asp-Val-Asp (SEQ ID NO: 3), or any subset thereof, from Trichomonas vaginalis (T2) for Glu-Leu-Lys (SEQ ID NO: 4), or any subset thereof, from Pseudomonas putida;

Detailed Description Text (32):
(c) Cys-His-Val-Val (SEQ ID NO: 5), or any subset thereof, from Trichomonas vaginalis (T2) for Ala-Leu-Gln-Leu (SEQ ID NO: 6), or any subset thereof, from Pseudomonas putida;

Detailed Description Text (33):
(d) Cys-Glu-Asn-Val-Gln-Asp-Ile-Ile-Asp-Asp (SEQ ID NO: 7), or any subset thereof, from Trichomonas vaginalis (T2) for Gly-Leu-Glu-Asp-Ile-Asp-Asp-Leu-Leu-Ala (SEQ ID NO: 8), or any subset thereof, from Pseudomonas putida; and

Detailed Description Text (45):
In a preferred example of the invention, such novel enzymes are patterned on homocysteinases found in various microorganisms including Pseudomonas, Clostridium, Aeromonas or Trichomonas, and particularly preferred homocysteinases are derived from T. vaginalis, including that expressed from the mgl1 gene (SEQ ID NO:11), or the mgl2 gene thereof.

Detailed Description Text (58):
In a representative example, the homocysteinase is patterned on an enzyme from Pseudomonas, Clostridium, Aeromonas or Trichomonas wherein one or more peptide (sub) (sub)sequences of the original polypeptide sequence(s) thereof are correspondingly replaced by one or more homologous peptide sequences of SEQ ID NO:10 that are selected from the group consisting of:

Detailed Description Text (87):
An additional enzyme useful in the practice of the double enzymatic subtraction approach is cystathionine .beta.-synthase, which catalyzes production in the body of cystathionine from homocysteine and serine. For blood serum or plasma samples, the reaction may be driven in the direction of cystathionine by taking advantage of the relatively high concentration of serine in the blood. Suitable sources of this enzyme include material isolated from Trichomonas, from Pseudomonas, or from mammalian tissues including, for example, liver. The enzyme may be used in an amount that provides a units/ml equivalent to the range described above for S-adenosylhomocysteine hydrolase. As mentioned above in connection with the use of SAHH, it is preferable that steps be taken to prevent this enzymatic reaction from running backwards, which may lead to inaccurate data. The reaction may be driven, and then maintained, in the direction of cystathionine by adding exogenous serine to the reaction samples, or by irreversibly inhibiting the enzyme once conversion to cystathionine has been accomplished. Suitable inhibitors include serine analogs of high affinity for the active site.

Detailed Description Text (192):
In these protocols, validations were first performed to confirm assay reproducibility. Accordingly, in a first test, separate tubes of serum (1:1 diluted and deproteinized) were spiked with different amounts of homocysteine (to attain final concentrations of 1 .mu.M, 5 .mu.M, 10 .mu.M, 15 .mu.M, and 20 .mu.M). A 50 .mu.l aliquot of homocysteinase (either the Trichomonas vaginalis enzyme expressed from pAC2-1, SEQ ID NO: 10, or wild type Pseudomonas pudita enzyme), was then added to each tube, and each tube was incubated at 37.degree. C. for 20 minutes with the lid tightly closed (to retain H.sub.2 S). The reaction was stopped by addition of 100 .mu.l of 1M NaOH, with mixing, followed immediately by a further addition of 0.3 ml of trapping solution (solution A), rapid mixing, and then centrifugation at 13,000 rpm for 1 minute (solution A was made by dissolving 4 g NaOH, 2.94 g trisodium citrate, and 10 g zinc acetate in 1 liter of water). The supernatant in each tube was then discarded, and the pellets were dissolved in 0.15 ml of solution B (2.7% w/v FeCl.sub.3 in 6N HCl) followed by addition of 0.75 ml solution C (0.2% w/v NDPD in 1N HCl). The contents were mixed thoroughly, followed by incubation at room temperature for 30 minutes, again with the lids closed. The concentration of product methylene blue, which is reflective of H.sub.2 S produced in the homocysteinase reaction, was read at 740 nm in a spectrophotometer. Linearity of the result was good for both the recombinant Pseudomonas putida and Trichomonas vaginalis enzymes.

Detailed Description Text (193):
A similar set of assays was performed in which serum was spiked to achieve a cysteine concentration of between 1 and 20 micromolar. In comparing the outcome for a recombinantly produced Trichomonas vaginalis enzyme (SEQ ID NO:10, from clone pAC2-1) and a recombinantly produced wild type Pseudomonas pudita enzyme, the experiment demonstrated that in spite of the high activities both enzymes show toward homocysteine, the pAC2-1 variant has much less activity toward cysteine and is therefore preferred.

Other Reference Publication (1):
H.Hori, et al., "Gene Cloning and Characterization of Pseudomonas putida L-Methionine-.alpha.-deamino-.gamma.-mercaptomethane-lyase", Cancer Research (1996) 56:2116-2122.

Other Reference Publication (9):
Esaki, N. et al., "L-Methionine gamma-Lyase from Pseudomonas putida and Aeromonas"in Methods in Enzymology (1987) 143:459-465.

CLAIMS:

1. A diagnostic kit for use in a single enzyme assay of the homocysteine concentration in a biological fluid of a subject, said kit comprising:

(a) a homocysteinase enzyme; and

(b) at least one reagent capable of being used to determine the amount of product hydrogen sulfide formed by reaction of homocysteinase on homocysteine; wherein

said homocysteinase has the property that at least about 90% of the hydrogen sulfide produced by action of said homocysteinase upon contacting a biological fluid in an assay for homocysteine is contributed by said homocysteine, when the concentrations of homocysteine and cysteine in said fluid are, respectively, about 20 .mu.molar and about 300 .mu.molar respectively, and

wherein said homocysteinase is that of Pseudomonas, Clostridium, Aeromonas or Trichomonas wherein one or more peptide sequences of such enzyme are correspondingly replaced by a sequence selected from the group consisting of residues 43-51, residues 168-176 and residues 304-312 of SEQ ID NO:10.

2. The diagnostic kit of claim 1 further comprising one or more reagents selected from the group consisting of:

(a) an agent capable of reducing disulfide bonds;

(b) a source of ferric iron;

(c) an N,N-dialkyl-p-phenylenediamine; and

(d) homocysteine as a calibration standard.

4. The diagnostic kit of claim 1 wherein said homocysteinase consists essentially of the amino acid sequence of Pseudomonas putida homocysteinase into which amino acid sequence portions of T. vaginalis homocysteinase have been inserted or wherein said homocysteinase consists essentially of the amino acid sequence of P. putida homocysteinase having portions of said ano acid sequence replaced by portions of T. vaginalis homocysteinase.

5. The diagnostic kit of claim 4 wherein the amino acid sequence Val-Gly-Ser-Gin-Ala-Leu-Val-Asp-Arg-Ile-Arg-Leu (SEQ ID NO:2) or fragment thereof from P. putida is replaced by the amino acid sequence Cys-Ser-Arg-Ala-Asp-lie-le-Ala-Lys-Val-Lys-Ser (SEQ ID NO: 1) or a corresponding fragment thereof from T. vaginalis (T2); or

wherein the amino acid sequence Glu-Leu-Lys (SEQ ID NO: 4) or fragment thereof of P. putida homocysteinase is replaced by Asp-Val-Asp (SEQ ID NO, 3) or the corresponding portion thereof from T. vaginalis (T2); or

wherein the amino acid sequence Ala-Leu-Gln-Leu (SEQ ID NO: 6) or fragment thereof of P. putida homocysteinase is replaced by Cys-His-Val-Val (SEQ ID NO: 5) or the corresponding portion thereof from T. vaginalis (T2); or

wherein the amino acid sequence Gly-Leu-Glu-Asp-le-Asp-Asp-Leu-Leu-Ala (SEQ ID NO: 8) or fragment thereof of P. putida homocysteinase is replaced by Cys-Glu-Asn-Val-Gln-Asp-Ile-Ile-Asp-Asp (SEQ ID NO: 7) or the corresponding portion thereof from T. vaginalis (T2); or

a combination of any of the replacements set forth above.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

☐ 12. Document ID: US 5994306 A

L11: Entry 12 of 25 File: USPT Nov 30, 1999

DOCUMENT-IDENTIFIER: US 5994306 A
**** See image for Certificate of Correction ****
TITLE: Fine-tuned protegrins

Drawing Description Text (5):
FIG. 4 is a graphical representation of the effect of serial transfer into antibiotic-containing media on the development of drug resistance in methicillin resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa.

Detailed Description Text (148):
Among Gram-negative organisms against which the protegrins are effective are Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Serratia marcescens, Haemophilus influenzae, Salmonella typhimurium, Acinetobacter calocoaceticus, C. pneumoniae, and Neisseria meningitidus, as well as other species including those within the genera represented above. For example, Neisseria gonorrhoeae is associated with sexually transmitted diseases (STDS) as is Chlamydia trachomatis. Also among the Gram-negative organisms are the gastric pathogens Helicobacter pylori, H. felis, and Campylobacter jejuni.

Detailed Description Text (178):
In treating Pseudomonas infection, applicants have found the following amides to be effective: RGGRLCYCRRRFCVCVGR(SEQ ID NO:1) - RGGRLCYCRPRFCVCVGR(SEQ ID NO:239) - RGGGLCYTRPRFTVCVGR(SEQ ID NO:228)

Detailed Description Text (216):
The protegrins have also been tested against various other organisms and show broad spectrum activity. In addition to their effectiveness in inhibiting the growth of or infection by microorganisms associated with STDs, the protegrins show strong activity against the following microorganisms in addition to those tested hereinabove: Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium, Staphylococcus aureus, Histoplasma capsulatum, Myobacterium avium-intracellulare, and Mycobacterium tuberculosis. The protegrins showed only fair activity against Vibrio vulnificus and were inactive against Vibrio cholerae and Borrelia burgdorferi.

Detailed Description Text (315):
Microorganisms: Escherichia coli ML-35p and vancomycin-resistant Enterococcus faecium (VRE) were obtained from Dr. Robert Lehrer (UCLA, see also, Lehrer et al., 1988, J. Immunol. Methods 108:153) and Dr. Gary Schoolnik (Stanford), respectively. Pseudomonas aeruginosa (ATCC 9027), Candida albicans (ATCC 1023), and methicillin resistant Staphylococcus aureus (ATCC 33591) were obtained from the American Type Culture Collection, Rockville, Md.

Detailed Description Text (399):

Tables 22-25 show activity in the kinetic bactericidal assay described above provided in terms of log reduction in CFUs for various peptides of the invention against MRSA, Pseudomonas aeruginosa, and the endogenous flora in saliva.

Detailed Description Paragraph Table (31):
TABLE 24 Log Reduction of Pseudomonas aeruginosa (ATCC 9027) CFUs after exposure to peptide (0.12 .mu.g/ml) in LTM medium at 37.degree. Sequence 15 min 120 min
RGGRLCYCRRRFCVCVGR 3.48 3.68 - RGWGLCYCRPRFCVCVGR 3.48 3.68 - RGGGLCYTRPRFTVCVGR 0.75 3.68 - RGGGLCYARKGFAVCVGR 1.20 3.68 - GWRLCYCRPRFCVCVGR 2.70 2.21 - RGGRLCYCRRRFCVC 2.25 >3.73 - LCYCRRRFCVCV 1.35 2.13
Initial inoculum approximately 4 .times. 10.sup.5 CFUs/ml.

Detailed Description Paragraph Table (35):
TABLE 29
MICS (.mu.g/ml) in Mueller Hinton Media RGGLCYCRGRFCVCVGR PG-1 Medium (amide) PG-1 (amide) (acid) Organism Suppl. - HSA + HSA - HSA + HSA - HSA

Staphylococcus aureus None 4 4 4 MSSA Staphylococcus aureus None 13.3 2 16 5.3 16 MRSA Enterococcus faecium None 2 0.33 1.3 0.25 2 VREF - hematin 1 + hematin 8 Bacillus subtilis None 0.7 0.8 0.2 Streptococcus - hematin 2 pneumoniae* + hematin 8 Streptococcus - hematin 0.12 salivarius* + hematin 0.5 viridans group Pseudomonas aeruginosa None 4 1.33 5.3 0.33 9.3 Klebsiella pneumoniae None 5.3 4 4 Serratia marcescens None 16 16 21 Escherichia coli None 4 0.33 5.3 0.12 4 - hematin 0.5 + hematin 8 Haemophilus influenzae* - hematin No growth + hematin 8 Acinetobacter None 2 3 4 calocoaceticus Neisseria meningitidis* - hematin 8 + hematin 32 Candida albicans None 8 4 16 8 16

CLAIMS:

2. The antimicrobial peptide of claim 1, wherein said peptide contains two disulfide bridges and is in the native form.

3. The antimicrobial peptide of claim 1, wherein said peptide contains one disulfide bridge and is in the bullet or kite form.

4. The antimicrobial peptide of claim 1, wherein said peptide contains no disulfide bridges and is in the snake form.

30. A recombinant expression system for production of the antimicrobial peptide according to claim 1, which expression system comprises a nucleotide sequence encoding said peptide operably linked to control sequences for effecting expression. expression.

31. A recombinant host cell, or progeny thereof, modified to contain the expression system of claim 30.

40. The method of claim 37, wherein the microbial infection is caused by Pseudomonas.

☐ 13. Document ID: US 5980895 A

L11: Entry 13 of 25 File: USPT Nov 9, 1999

DOCUMENT-IDENTIFIER: US 5980895 A
TITLE: Immunotoxin containing a disulfide-stabilized antibody fragment joined to a Pseudomonas exotoxin that does not require proteolytic activation

Abstract Text (1):
This invention provides for immunotoxins comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic activity attached to an Fv antibody fragment having a variable heavy chain region bound through at least one disulfide bond to a variable light chain region. The combination of a "disulfide-stabilized" binding agent fused to a PE that does not require proteolytic activation provides an immunotoxin having surprising cytotoxic activity.

Brief Summary Text (2):
This invention pertains to the production and use of Pseudomonas-derived immunotoxins modified to increase their toxicity and potency in therapy. In particular, the immunotoxins of this invention include a disulfide-stabilized (ds) target-binding agent, such as the variable region of an antibody molecule, and a Pseudomonas exotoxin that does not require proteolytic activation for cytotoxic activity.

Brief Summary Text (3):
Immunotoxins were initially produced by chemically coupling antibodies to toxins (Vitetta et al. Cell, 41: 653-654 (1985); Pastan et al., Ann. Rev. Biochem. 61: 331-354 (1992)) to form chimeric molecules. In these molecules, the antibody portion mediated selective binding to target cells, while the toxin portion mediated translocation into the cytosol and subsequent cell killing. Several toxins have been used to make immunotoxins, including ricin A chain, blocked ricin, saporin, pokeweed antiviral protein, diphtheria toxin and Pseudomonas exotoxin A (PE) (Pastan et al., Science 254: 1173-1177 (1991); Vitetta et al., Semin. Cell Biol. 2: 47-58 (1991); Tazzari et al., Br. J. Hematol. 81: 203-211 (1992); Uckun et al., Blood, 79: 2201-2214 (1992)).

Brief Summary Text (8):
The present invention is premised, in part, on the discovery that immunotoxins comprising both a disulfide-stabilized binding agent and a Pseudomonas exotoxin modified so that proteolytic cleavage is not required for cytotoxicity, show cytotoxicity far greater than would be expected based on the performance of fusion proteins comprising either the disulfide stabilized binding protein or the modified Pseudomonas exotoxin alone.

Brief Summary Text (9):
Thus, in one embodiment, this invention provides for an immunotoxin comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic cytotoxic activity attached to a variable heavy (V.sub.H) region of an Fv antibody fragment where the variable heavy region is bound through at least one disulfide bond to a variable light (V.sub.L) chain region. In a preferred embodiment, the Pseudomonas exotoxin is a truncated Pseudomonas exotoxin lacking domain Ia. In another embodiment, Pseudomonas exotoxin lacks residues 1 through 279. The variable heavy chain region can substantially replace domain Ib of the Pseudomonas exotoxin, or alternatively, it can be located in the carboxyl terminus of the Pseudomonas exotoxin. The amino terminus of the heavy chain region can be attached to the PE through a peptide linker (e.g. SGGGGS (SEQ ID NO:10)). The carboxyl terminus of the

heavy chain region can also be attached to the PE through a peptide linker (e.g., KASGGPE (SEQ ID NO:11)). In a preferred embodiment, the antibody fragment is from B1, B3, B5, e23, BR96, anti-Tac, RFB4, or HB21, more preferably from B1, B3, B5, and and e23. The carboxyl terminal sequence of the immunotoxin can be KDEL (SEQ ID NO:9). Particularly preferred immunotoxins include PE35/e23(dsfv)KDEL and B1(dsFv) PE33.

Brief Summary Text (10):
In another embodiment, the variable light (V.sub.L) region rather than the variable heavy region (V.sub.H) is attached (fused) to the Pseudomonas exotoxin, while the variable heavy (V.sub.H) chain is bound to the variable light (V.sub.L) chain through at least one disulfide bond. Particularly preferred embodiments include all of the embodiments described above differing only in that the V.sub.L chain is substituted for the V.sub.H chain and vice versa.

Brief Summary Text (11):
This invention also provides for nucleic acids encoding all of the above-described immunotoxins. Thus, in one embodiment, this invention provides for a nucleic acid encoding an immunotoxin comprising a heavy chain variable region of an Fv antibody fragment attached to a Pseudomonas exotoxin that does not require proteolytic activation for cytotoxic activity. The encoded heavy chain variable region contains cysteine residues that form disulfide linkages with a variable light chain region of an Fv fragment and the antibody fragments comprise the variable light or variable heavy chains of B1, B3, B5, e23, BR96, anti-Tac, RFB4, or HB21. In a preferred embodiment, the nucleic acid encodes an immunotoxin in which the heavy chain variable region is substituted for domain Ib of the Pseudomonas exotoxin. In another embodiment, the nucleic acid encodes an immunotoxin in which the heavy chain variable region is located after residue 607 of the Pseudomonas exotoxin. The PE component of the encoded immunotoxin preferably lacks amino acid residues 1 through 279. In another preferred embodiment, this invention also provides for nucleic acids as described above encoding immunotoxins in which the V.sub.L chain is substituted for the V.sub.H chain and vice versa.

Brief Summary Text (12):
It was also a discovery of this invention that single chain immunotoxins comprising V.sub.L or V.sub.H regions alone, rather than as components of Fv fragments, are capable of binding their target molecules. Thus, in yet another embodiment, this invention provides for a single chain immunotoxin fusion protein comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic cytotoxic activity attached to a variable light (V.sub.L) or a variable heavy (V.sub.H) chain region. Suitable toxin components include any of the Pseudomonas exotoxins described above. In a preferred embodiment, the Pseudomonas exotoxin is a truncated Pseudomonas exotoxin lacking domain Ia. In another preferred embodiment, the Pseudomonas exotoxin lacks residues 1 through 279. The variable heavy or light chain can substantially replace domain Ib, or can be located in the carboxyl terminus of the Pseudomonas exotoxin. The amino terminus of the variable heavy or light chain region can be attached to the PE through a peptide linker (e.g., SGGGGS) while the carboxyl terminus of the variable heavy or light chain region can be attached to the PE through a peptide linker (e.g., KASGGPE). The variable heavy or light chain are preferably derived from B1, B3, B5, e23, BR96, anti-Tac, RFB4, or HB21, and more preferably from B1, B3, B5 and e23. The immunotoxin can have the carboxyl terminal sequence KDEL.

Brief Summary Text (15):
The immunotoxins of this invention are suitable for use in pharmacological compositions. This invention thus provides for a pharmaceutical composition comprising an effective amount of an immunotoxin in a pharmacologically acceptable excipient. Preferred immunotoxins include any of the above-described immunotoxins comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic activity attached to a heavy chain region of an Fv antibody fragment

which is bound through at least one disulfide bond to a variable light chain region or, conversely, attached to a light chain region of an Fv antibody fragment which is is bound through at least one disulfide bond to a variable heavy chain region.

Brief Summary Text (16):
Finally, this invention also provides methods of delivering an antibody to the cytosol of a cell. The methods involve contacting the cell with a chimeric molecule comprising the antibody attached to a Pseudomonas exotoxin that does not require proteolytic cleavage for translocation into the cytosol of said cell. The chimeric molecule is preferably a fusion protein in which the antibody (e.g., a V.sub.H or a V.sub.L region) is substituted into domain Ib, domain II or the carboxyl terminus of domain III. Domain III is preferably inactivated (its cytotoxic activity substantially eliminated) by truncation, mutation, or insertion of a heterologous peptide sequence.

Detailed Description Text (2):
This invention relates to Pseudomonas exotoxin (PE) based immunotoxins having increased cytotoxic activity. It was a surprising discovery of the present invention that immunotoxins comprising a disulfide-stabilized binding agent attached to a Pseudomonas exotoxin that has been modified so that proteolytic cleavage is not required for cytotoxic activity show unexpected high levels of cytotoxicity, particularly greater than a ten-fold increase in cytotoxicity to target cells. This cytotoxicity combined with the smaller size of the immunotoxin which provides greater penetration into solid tumors results in an immunotoxin of improved pharmacological efficacy. The term binding agent, as used herein, refers to a molecule that specifically recognizes and binds to a particular preselected target molecule. The binding agent is thus capable of specifically targeting cells that express the preselected target molecule. Thus chimeric immunotoxins including a binding agent specifically bind to and kill or inhibit growth of cells bearing target molecules recognized by the binding agent.

Detailed Description Text (5):
As indicated above, the disulfide-stabilized binding agent is attached to a Pseudomonas exotoxin which is modified so that it is cytotoxic without requiring proteolytic activation. As explained below in Section III, this typically entails truncating the amino terminus to at least position 279. Methods of producing Pseudomonas exotoxins that do not require proteolytic cleavage for activation are described in copending patent application Ser. No. 08/405,615, filed on Mar. 15, 1995 which is a continuation of Ser. No. 07/901,709 filed on Jun. 18, 1992.

Detailed Description Text (6):
The disulfide-stabilized binding agent may be located at virtually any position within the modified Pseudomonas exotoxin. In one preferred embodiment, the binding agent is inserted in replacement for domain Ia as has been accomplished in what is known as the TGF.alpha./PE40 molecule (also referred to as TP40) described in Heimbrook et al., Proc. Natl. Acad. Sci., USA, 87: 4697-4701 (1990) and in commonly assigned U.S. Ser. No. 07/865,722 filed Apr. 8, 1992 and in U.S. Ser. No. 07/522,563 filed May 14, 1990.

Detailed Description Text (11):
It was also a discovery of the present invention that variable heavy or light chain regions alone, rather than as a component of an Fv region, are capable of specifically binding to their target molecules. Thus, in one embodiment, this invention provides for single chain immunotoxin fusion proteins comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic cytotoxic activity attached to a variable light (V.sub.L) or a variable heavy (V.sub.H) chain region. In effect these fusion proteins are made in the same manner as the disulfide-stabilized fusion proteins described above, but the step whereby the respective variable regions are joined by disulfide linkages is omitted. In addition, as no disulfide linkages need be formed, there is no need to introduce

cysteine into either of the variable regions, or to eliminate cysteines existing in the PE. Either the variable light chain or the variable heavy chain can be expressed expressed in fusion with the modified PE.

Detailed Description Text (56):
Separate vectors with sequences for the desired V.sub.H and V.sub.L sequences (or other homologous V sequences) may be made from the mutagenized plasmid. The sequences encoding the heavy chain regions and the light chain regions are produced and expressed in separate cultures in any manner known or described in the art, with the exception of the guidelines provided below. If another sequence, such as a sequence for a toxin, is to be incorporated into the expressed polypeptide, it can be linked to the V.sub.H or the V.sub.L sequence at either the N- or C-terminus or be inserted into other protein sequences in a suitable position. For example, for Pseudomonas exotoxin (PE) derived fusion proteins, either V.sub.H or V.sub.L should be linked to the N-terminus of the toxin or be inserted into domain III of PE, like for example TGF.alpha. in Theuer et al., J. Urol., 149: 1626-1632 (1993), or inserted in place of domain Ib of PE. For Diphtheria toxin-derived immunotoxins, V.sub.H or V.sub.L is preferably linked to the C-terminus of the toxin.

Detailed Description Text (64):
As indicated above, the preferred immunotoxins comprise a disulfide-stabilized binding agent joined to a Pseudomonas exotoxin modified (e.g. truncated) so that proteolytic cleavage is not required for cytotoxic activity. As used herein, cytotoxic activity refers to the ability to kill a cell or to significantly reduce its growth or proliferation rate.

Detailed Description Text (75):
The fusion proteins of this invention can be produced according to a number of means well known to those of skill in the art. Where the disulfide-stabilized binding agent and/or the modified Pseudomonas exotoxin are relatively short (i.e., less than about 50 amino acids) they may be synthesized using standard chemical peptide synthesis techniques. Where both molecules are relatively short the chimeric molecule may be synthesized as a single contiguous polypeptide. Alternatively the targeting molecule and the effector molecule may be synthesized separately and then fused by condensation of the amino terminus of one molecule with the carboxyl terminus of the other molecule thereby forming a peptide bond. Alternatively, the targeting and effector molecules may each be condensed with one end of a peptide spacer molecule thereby forming a contiguous fusion protein.

Detailed Description Text (104):
Compositions for the intracellular delivery of the antibody are preferably fusion proteins formed by joining a Pseudomonas exotoxin to an antibody fragment, more preferably to a V.sub.H or a V.sub.L antibody fragment. The Pseudomonas exotoxin is preferably truncated, but still includes a functional translocation domain (domain II).

Detailed Description Text (114):
In order to construct an active recombinant immunotoxin that was smaller than the current generation of recombinant immunotoxins and that did not need intracellular proteolytic cleavage for activation, the antibody fragment B1(dsFv) was inserted between domains II and III of a Pseudomonas exotoxin. This was accomplished by substituting B1(dsFv) for domain Ib of PE37, a truncated form of PE that contains only the portion of the toxin that undergoes translocation to the cytosol. In particular, B1(V.sub.H)R44C was inserted after amino acid 364 of PE and the insert was preceded by a small flexible peptide linker GGGGS (SEQ ID NO:12). Following the V.sub.H domain was another peptide, KASGGPE (SEQ ID NO:11) (C3 connector) (Brinkmann et al., Proc. Natl. Acad. Sci. USA, 89: 3075-3079 (1992)), connecting the carboxyl terminus of V.sub.H to amino acid 395 of the Pseudomonas exotoxin.

Other Reference Publication (4):

Theuer, Charles P., et al. (1993) "A Recombinant Form of Pseudomonas Exotoxin A Containing Transforming Growth Factor Alpha Near Its Carboxyl Terminus For The Treatment of Bladder Cancer", The Journal of Urology, 149:1626-1632.

Other Reference Publication (5):
Theuer, Charles P., et al. (1993) "Immunotoxins Made with a Recombinant Form of Pseudomonas Exotoxin A That Do Not Require Proteolysis for Activity", Cancer Research, 53:340-347.

Other Reference Publication (50):
Robert J. Kreitman, et al., "Pseudomonas Exotoxin-based Immunotoxins Containing the Antibody LL2 or LL2-Fab Induce Regression of Subcutaneous Human B-Cell Lymphoma in Mice," 819-825 Cancer Research (Feb. 15, 1993).

Other Reference Publication (54):
Elizabeth Mansfield, et al., "Characterization of RFB4-Pseudomonas Extotoxin A Immunotoxins Targeted to CD22 on B-Cell Malignancies," 557-563 Bioconjugate Chemistry, (Sep. 5, 1996).

CLAIMS:

1. An immunotoxin that binds to an epitope on a target cell and that does not require proteolytic activation for cytotoxic activity, said inmunotoxin comprising a Pseudomonas exotoxin (PE) lacking amino acids 1 through 279 attached to a variable variable heavy (V.sub.H) chain framework region of an Fv antibody fragment wherein said variable heavy chain region is bound through at least one disulfide bond to a variable light (V.sub.L) chain framework region thereby forming a double-stranded Fv region (dsFv) and further wherein said variable heavy chain region or said variable light chain region replaces half or more of domain Ib of said Pseudomonas exotoxin.

2. The immunotoxin of claim 1, wherein the amino terminus of the heavy chain region is attached to the PE through a peptide linker.

4. The immunotoxin of claim 1, wherein the carboxyl terminus of the heavy chain region is attached to the PE through a peptide linker.

7. The immunotoxin of claim 1, having KDEL (SEQ ID NO:9) as the carboxyl terminal sequence of said PE.

12. An immunotoxin comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic activity attached to a variable light (V.sub.L) chain region of an Fv antibody fragment wherein said variable light chain region is bound through at least one disulfide bond to a variable heavy (V.sub.H) chain region said Pseudomonas exotoxin is lacking amino acids 1 through 279, and said variable light (V.sub.L) chain region replaces half or more of domain Ib of said Pseudomonas exotoxin.

13. The immunotoxin of claim 12, wherein the amino terminus of the light chain region is attached to the PE through a peptide linker.

15. The immunotoxin of claim 12, wherein the carboxyl terminus of the light chain region is attached to the PE through a peptide linker.

18. The immunotoxin of claim 12, having KDEL (SEQ ID NO:9) as the carboxyl terminal sequence of said PE.

19. A single chain immunotoxin fusion protein, said fusion protein comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic cytotoxic activity, said immunotoxin comprising a Pseudomonas exotoxin lacking

amino acids 1 through 279 and in which half or more of domain Ib is replaced with either a variable light (V.sub.L) or a variable heavy (V.sub.H) chain region of an antibody, wherein said immunotoxin does not contain both a variable light (V.sub.L) and a variable heavy (V.sub.H) region.

20. The immunotoxin of claim 19, wherein an amino terminus of the variable heavy or variable light chain region is attached to the PE through a peptide linker.

22. The immunotoxin of claim 20, wherein a carboxyl terminus of the variable heavy or variable light chain region is attached to the PE through a peptide linker.

25. The immunotoxin of claim 19, having KDEL (SEQ ID NO:9) as the carboxyl terminal sequence of said PE.

26. A pharmaceutical composition comprising an effective amount of an immunotoxin in a pharmacologically acceptable excipient, the immunotoxin comprising a Pseudomonas exotoxin (PE) that does not require proteolytic activation for cytotoxic cytotoxic activity attached to an Fv antibody fragment having a variable heavy chain framework region bound through at least one disulfide bond to a variable light chain framework region, wherein half or more of domain Ib of said Pseudomonas exotoxin is replaced by either a V.sub.H or a V.sub.L domain of said Fv antibody.

27. The composition of claim 1, wherein half or more of PE domain Ib is replaced by a V.sub.H domain of said Fv antibody.

28. The composition of claim 1, wherein half or more of PE domain Ib is replaced by a V.sub.L domain of said Fv antibody.

29. The composition of claim 26, wherein half or more of PE domain Ib is replaced by a V.sub.H domain of said Fv antibody.

30. The composition of claim 26, wherein half or more of PE domain Ib is replaced by a V.sub.L domain of said Fv antibody.

☐ 14. Document ID: US 5965406 A

L11: Entry 14 of 25 File: USPT Oct 12, 1999

DOCUMENT-IDENTIFIER: US 5965406 A
**** See image for Certificate of Correction ****
TITLE: Recombinant DNAS encoding three-part hybrid proteins

Brief Summary Text (7):
The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps: (i) the binding domain of diphtheria toxin binds to specific receptors on the surface of a sensitive cell; (ii) while bound to its receptor, the toxin molecule is internalized into an endocytic vesicle; (iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage in l.sub.1 between fragments A and B; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin spontaneously inserts into the endosomal membrane; (v) once embedded in the membrane, the translocation domain of the toxin facilitates the delivery of Fragment A into the cytosol; (vi) the catalytic activity of Fragment A (i.e., the

nicotinamide adenine dinucleotide--dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the intoxicated cell. It is apparent that a single molecule of Fragment A introduced into the cytosol is sufficient to shut down the cell's protein synthesis machinery and kill the cell. The mechanism of cell killing by Pseudomonas exotoxin A, and possibly by certain other naturally-occurring toxins, is is very similar.

Brief Summary Text (11):
(b) the second part including a portion of a translocation domain of a protein, provided that (i) the hybrid molecule does not include an enzymatically-active portion of such protein, (ii) the first part and the second part are not segments of the same naturally-occurring polypeptide toxin, and (iii) the portion of the translocation domain, when covalently bonded to an enzymatically-active portion (i.e., the "effector region") of a toxin selected from diphtheria toxin, Pseudomonas exotoxin A, cholera toxin, ricin toxin, and Shiga-like toxin, is capable capable of translocating such effector region across the cytoplasmic membrane of the cell. "Translocation" here means the facilitation of movement of a chemical entity from the exterior surface of a cellular membrane (or what constituted the exterior surface prior to formation of an endocytic vesicle), through the membrane, and into the cytosol at the interior of the cell. A "translocation domain" is a segment of a protein which, when the protein is bound to the exterior surface of a cellular membrane, is capable of translocating some portion of that protein through the membrane.

Brief Summary Text (16):
In preferred embodiments, the second part comprises at least a portion of the translocation domain of a naturally-occurring toxin (e.g. diphtheria toxin or Pseudomonas exotoxin A), and the ligand comprises a hormone (e.g. a polypeptide hormone such as insulin, Interleukin II (also termed "IL2"), Interleukin IV, Interleukin VI or EGF, or, alternatively, a steroid hormone); an antigen-binding, single-chain analog of a monoclonal antibody; or a polypeptide toxin capable of binding to the desired class of cells (more preferably, both the first and the second parts are derived from diphtheria toxin); where both the first and second parts are polypeptides, the hybrid molecule is preferably a recombinant protein; the hybrid molecule preferably additionally comprises a third part which is connected to the second part by at least one covalent bond and which is a chemical entity to be introduced into the cell (provided that where the third part is a polypeptide, the cleavable bond is a disulfide bond). More preferably, all three parts are polypeptides and the hybrid molecule is a recombinant protein (that is, a protein produced by recombinant DNA techniques); the third part and the second part are linked through a proteolytically-sensitive disulfide loop (defined below); the third part is an antigen-binding, single-chain analog of a monoclonal antibody (where such antigen is, for example, a viral protein such as the human immunodeficiency virus (HIV) protease), or alternatively, the enzymatically active portion of an enzyme (e.g., hexosaminidase A; .alpha.-1,4-glucosidase; phenylalanine hydroxylase; a protease; a nuclease; or a toxin such as cholera toxin, LT toxin, C3 toxin, Shiga toxin, E.coli Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin or Pseudomonas exotoxin A), and most preferably it supplies an enzymatic activity in which the cell is deficient, as, for example, in the case of a genetic deficiency. Where the enzyme is cholera toxin, the resulting hybrid molecule may be used to raise the cyclic AMP level within an animal cell: preferably, the cell so treated is a T-cell and the hybrid molecule includes at least a portion of the binding domain of IL2. By "proteolytically-sensitive disulfide loop" is meant a sequence of at least 5 amino acid residues (preferably from 6 to 30, and more preferably from 11 to 18) joined in series by-peptide bonds, the first and last residues of which sequence are cys residues which link to form a cystine disulfide bond. At least two of the remaining residues of the sequence together create a proteolytically-sensitive site: i.e., a peptide bond formed between two residues, the second (carboxyl side) of which may

be, e.g., Arg, Lys, Phe, Tyr, or Trp. There is preferably also at least one Ser residue within the sequence of the loop. The loop, which may be a naturally-occurring feature of the second part or the third part, or may be engineered (e.g., from a synthetic DNA sequence) into the hybrid, joins the third part to the second part by two types of covalent linkages, peptide and sulfhydryl, ensuring that these two portions of the hybrid will remain associated with each other, even in the presence of extracellular proteases, until after the hybrid has bound to the target cell, but will separate at the appropriate stage. Both the proteolytically-sensitive sensitive peptide bond(s) within the disulfide loop and the disulfide bond itself are cleaved at some point prior to or during passage of the chemical entity through the cellular membrane of the endocytic vesicle, resulting in the release of the chemical entity into the cytosol, free of the receptor-bound cell-binding ligand portion (the first part), and translocation domain portion (the second part) of the hybrid.

Brief Summary Text (22):
Based upon the observation that certain types of polypeptide toxins have three separate functional regions, one region which binds the molecule to particular receptors on the surface of a target cell, a second one which facilitates entry of the enzymatically-active region into the cytosol of the cell, and a third region which exhibits the enzymatic activity that characterizes the toxic effect of the molecule, the invention comprises bi- or tripartite hybrid molecules in which any of these regions may be replaced with functionally comparable regions from other sources. That is, the first functional region may be replaced with a particular binding moiety which binds the hybrid molecule to a selected class of cells, such as IL2 (which binds to high-affipity IL2 receptor-bearing T-cells), or a melanocyte stimulating hormone (aMSH, which binds to melanocytes), or a moiety which binds to a broad spectrum of cell types, as is characteristic of the binding domains of cholera toxin and diphtheria toxin; the second part may be taken from any type of polypeptide in which a translocation domain is identifiable, but will most likely be from a toxin molecule that translocates in a manner similar to diphtheria toxin and Pseudomonas exotoxin A. The optional third part may be any type of moiety that one wants to insert into the cell and that will fit through the channel in the membrane formed by the translocation domain: for example, a cell-killing enzyme such as Shiga toxin; a metabolic enzyme such as phenylalanine hydroxylase (the enzyme in which phenylketonurics are deficient); an antigen-binding, single-chain analog of a monoclonal antibody against an antigen that appears within the target cell; or a fluorescent label.

Detailed Description Text (3):
Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin and Pseudomonas exotoxin A, and may include other toxins and non-toxin molecules, as well. The translocation domains of diphtheria toxin and Pseudomonas exotoxin A are well characterized (see, e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82:1692-1696, 1985; Colombatti et al., J. Biol. Chem. 261:3030-3035, 1986; and Deleers et al., FEBS 160:82-86, 1983), and the existence and location of such a domain in other molecules may be determined by methods such as those employed by Hwang et al., Cell 48:129-136, 1987; and Gray et al., Proc. Natl. Acad. Sci. USA 81:2645-2649, 1984.

Detailed Description Text (57):
The translocation function of the hybrid molecule may be supplied by an appropriate piece of a polypeptide other than diphtheria toxin, but which is capable of translocating in a manner analogous to that of diphtheria toxin (e.g., Pseudomonas exotoxin A, botulinum, neurotoxin, or ricin), or in any other manner which accomplishes the objective of translocating the functional "third part" of the hybrid molecule into the cell's cytoplasm.

Detailed Description Text (60):
Likewise, the hybrid of the invention will be useful for specifically destroying

certain cells. Besides the cholera toxin A.sub.1 -hybrid, ricin A-hybrid and Shiga-like toxin A-hybrid exemplified above, a cell-killing function may be provided by the enzymatically-active portion of any polypeptide toxin, including but not limited limited to LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin and Pseudomonas exotoxin A. Cells to be targeted might include cancer cells, virus-infected cells, or adipocytes.

CLAIMS:

1. A recombinant DNA molecule encoding a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said naturally occurring protein of (b).

2. The recombinant DNA molecule of claim 1, wherein said first part comprises the binding domain of said cell-binding polypeptide ligand.

3. The recombinant DNA molecule of claim 1, wherein said first part comprises the cell-binding polypeptide ligand.

4. The recombinant DNA molecule of claim 1, wherein said cell-binding polypeptide binding ligand is a hormone.

5. The recombinant DNA molecule of claim 1, wherein said cell-binding polypeptide ligand is an antigen-binding, single-chain analog of a monoclonal antibody.

6. The recombinant DNA molecule of claim 1, wherein said first part comprises a monoclonal antibody.

7. The recombinant DNA molecule of claim 1, wherein said first part comprises a portion of the binding domain of a polypeptide toxin.

8. The recombinant DNA molecule of claim 1, wherein said polypeptide entity of (c) is an antigen-binding, single-chain analog of a monoclonal antibody.

9. The recombinant DNA molecule of claim 1, wherein said polypeptide entity of (c) comprises an enzymatically active portion of an enzyme.

10. The recombinant DNA molecule of claim 1, wherein said polypeptide entity of (c) comprises an enzymatically active portion of a protease.

11. The recombinant DNA molecule of claim 1, wherein said polypeptide entity of (c) comprises an enzymatically active portion of a nuclease.

12. The recombinant DNA molecule of claim 1, wherein said polypeptide entity of (c) comprises an enzymatically active portion of a toxin.

13. The recombinant DNA molecule of claim 1, wherein said second and third parts are

are connected via a proteolytically-sensitive disulfide loop.

14. The recombinant DNA molecule of claim 1, wherein said second part comprises a portion of the translocation domain of Shiga-like toxin.

15. The recombinant DNA molecule of claim 1, wherein said third part comprises an enzymatically active portion of Shiga-like toxin A, and wherein said second and third parts are connected via a proteolytically-sensitive disulfide-loop.

16. The recombinant DNA molecule of claim 1, wherein said first part is non-native with respect to said naturally occurring protein of (b).

17. A vector containing the recombinant DNA molecule of claim 1.

18. A cell transformed with the recombinant DNA molecule of claim 1.

19. The recombinant DNA molecule of claim 4, wherein said hormone is selected from the group consisting of insulin, interleukin II, interleukin IV, interleukin VI and epidermal growth factor (EGF).

20. The recombinant DNA molecule of claim 7, wherein said toxin is diphtheria toxin. toxin.

21. The recombinant DNA molecule of claim 12, wherein said toxin is selected from the group consisting of cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin, and Pseudomonas exotoxin A.

22. The recombinant DNA molecule of claim 15, wherein said first part comprises the binding domain of interleukin II.

24. The recombinant DNA molecule of claim 19, wherein said hormone is interleukin II.

25. The recombinant DNA molecule of claim 19, wherein said hormone is EGF.

26. The recombinant DNA molecule of claim 21, wherein said toxin is cholera toxin.

27. The recombinant DNA molecule of claim 21, wherein said toxin is Shiga-like toxin.

28. The recombinant DNA molecule of claim 21, wherein said toxin is ricin toxin.

29. A recombinant DNA molecule encoding a hybrid protein comprising a first part, a second part and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of the translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane and into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said polypeptide entity is non-native with respect to said diphtheria toxin.

30. The recombinant DNA molecule of claim 29, wherein said first part comprises a portion of the binding domain of interleukin II effective to cause said hybrid

protein to bind to an interleukin II receptor-bearing cell.

31. The recombinant DNA molecule of claim 29, wherein said first part comprises a portion of the binding domain of diphtheria toxin.

32. The recombinant DNA molecule of claim 29, wherein said first part comprises a portion of the binding domain of EGF.

33. The recombinant DNA molecule of claim 29, wherein said second part comprises Fragment B' of diphtheria toxin illustrated in FIG. 3.

34. The recombinant DNA molecule of claim 29, wherein said third part comprises an enzymatically active portion of cholera toxin.

35. The recombinant DNA molecule of claim 29, wherein said third part comprises an enzymatically active portion of ricin toxin.

36. The recombinant DNA molecule of claim 29, wherein said third part comprises an enzymatically active portion of Shiga-like toxin.

37. The recombinant DNA molecule of claim 29, wherein said cell binding polypeptide ligand is non-native to said diphtheria toxin.

38. The recombinant DNA molecule of claim 29, wherein said second and third parts are connected via a proteolytically-sensitive disulfide loop.

39. The recombinant DNA molecule of claim 29, which encodes the hybrid protein CTA/DTB'/IL2.

40. The recombinant DNA molecule of claim 29, which encodes the hybrid protein SLA/DTB'/IL2.

41. The recombinant DNA molecule of claim 29, which encodes the hybrid protein ricin ricin A/DTB'/IL2.

42. A vector containing the recombinant DNA molecule of claim 29.

43. A cell transformed with the recombinant DNA molecule of claim 29.

45. A method of preparing a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of a animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said naturally occurring protein of (b) comprising the steps of:

providing a cell transformed with a recombinant DNA molecule encoding the hybrid protein, and

culturing the transformed cell to allow expression of the recombinant DNA molecule

such that the hybrid protein is produced.

48. A method of preparing a hybrid protein comprising a first pail, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause the hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the the cell, wherein said third part is non-native with respect to said diphtheria toxin, comprising the steps of:

providing a cell transformed with a recombinant DNA molecule encoding the hybrid protein, and

culturing the transformed cell to allow expression of the recombinant DNA molecule such that the hybrid protein is produced.

☐ 15. Document ID: US 5942664 A

L11: Entry 15 of 25 File: USPT Aug 24, 1999

DOCUMENT-IDENTIFIER: US 5942664 A
**** See image for Certificate of Correction ****
TITLE: Bacillus thuringiensis Cry1C compositions toxic to lepidopteran insects and methods for making Cry1C mutants

Brief Summary Text (163):
In one preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension comprising lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferably the cells are B. thuringiensis cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein is contemplated to be useful, such as Bacillus spp., including B. megaterium, B. subtilis; B. cereus, Escherichia spp., including E. coli, and/or Pseudomonas spp., including P. cepacia, P. aeruginosa, and P. fluorescens. Alternatively, the oil flowable suspension may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (164):
In a second preferred embodiment, the bioinsecticide composition comprises a water dispersible granule or powder. This granule or powder may comprise lysed or unlysed bacterial cells, spores, or crystals which contain one or more of the novel crystal proteins disclosed herein. Preferred sources for these compositions include bacterial cells such as B. thuringiensis cells, however, bacteria of the genera

Bacillus, Escherichia, and Pseudomonas which have been transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Alternatively, the granule or powder may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Brief Summary Text (165):
In a third important embodiment, the bioinsecticide composition comprises a wettable powder, spray, emulsion, colloid, aqueous or organic solution, dust, pellet, or collodial concentrate. Such a composition may contain either unlysed or lysed bacterial cells, spores, crystals, or cell extracts as described above, which contain one or more of the novel crystal proteins disclosed herein. Preferred bacterial cells are B. thuringiensis cells, however, bacteria such as B. megaterium, B. subtilis, B. cereus, E. coli, or Pseudomonas spp. cells transformed with a DNA segment disclosed herein and expressing the crystal protein are also contemplated to be useful. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or alternatively, dissolve in a controlled-release, sustained-release, or other time-dependent manner. Alternatively, such a composition may consist of a combination of one or more of the following compositions: lysed or unlysed bacterial cells, spores, crystals, and/or purified crystal proteins.

Detailed Description Text (16):
The nucleotide sequences of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the sites of lepidopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B. thuringiensis toxin.

Detailed Description Text (17):
Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility or toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and Gram-positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium; Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae, Actinomycetales, and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

Detailed Description Text (19):
Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli,

Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Streptomyces lividans and the like.

Detailed Description Text (22):
A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera Bacillus, Pseudomonas, Erwinia, Serratia, Klebsiella, Zanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes; fungi, particularly yeast, e.g., genera Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phytosphere bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodobacter sphaeroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes eutrophus, and Azotobacter vinlandii; and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans.

Detailed Description Text (54):
In an alternate embodiment, the recombinant expression of DNAs encoding the crystal proteins of the present invention is performed using a transformed Gram-negative bacterium such as an E. coli or Pseudomonas spp. host cell. Promoters which function function in high-level expression of target polypeptides in E. coli and other Gram-negative host cells are also well-known in the art.

CLAIMS:

1. An isolated Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 4 and 5 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

2. The polypeptide of claim 1, wherein said loop region extends from about amino acid 148 to about amino acid 156 of said polypeptide.

14. A composition comprising a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 4 and 5 of domain 1, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

22. A method of preparing a Bacillus thuringiensis Cry1C endotoxin polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide, comprising:

(a) identifying a Cry1C .delta.-endotoxin polypeptide having a loop region between .alpha. helices 4 and 5 of domain 1 of said polypeptide;

(b) substituting at least one native amino acid in said loop region with at least one other amino acid; and

(c) obtaining the Cry1C .delta.-endotoxin polypeptide so produced.

30. The method of claim 22, wherein said loop region extends from about amino acid 148 to about amino acid 156 of said polypeptide.

35. A polynucleotide comprising an isolated gene that encodes a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 4 and 5 of domain 1 of said polypeptide, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

38. The polynucleotide of claim 35, wherein said loop region extents firm about amino acid 148 to about amino acid 156 of said polypeptide.

47. The polynucleotide of claim 35, wherein said gene is operably linked to a promoter that expresses said gene to produce said polypeptide.

50. The polynucleotide of claim 49, wherein said plant-expressible promoter is selected from the group consisting of corn sucrose synthetase 1, corn alcohol dehydrogenase 1, con light harvesting complex, corn heat shock protein, pea small subunit RuBP carboxylase, Ti plasmid opine synthase, Ti plaid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, Potato patatin, lectin, CaMV 35S, and the S-E9 small subunit RuBP carboxylase promoter.

51. A vector comprising at least one gene that encodes a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more no acid mutations in the loop region between .alpha. 4 and 5 of domain 1 of said polypeptide, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

55. A host cell comprising a gene encoding a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 4 and 5 of domain 1 of said polypeptide, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

58. The host call of claim 51, wherein said bacterial cell is an E. coli, Bacillus thuringiensis, Bacillus subtilis, Bacillus megaterium; Bacillus cereus, Agrobacterium or Pseudomonas cell.

63. A transgenic plant having incorporated into its genome a selected polynucleotide, said polynucleotide comprising a gene that encodes a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having one or more amino acid mutations in the loop region between .alpha. helices 4 and 5 of domain 1 of said polypeptide, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

64. The transgenic plant of claim 63, wherein said loop region extends from about amino acid 148 to about amino acid 156 of said polypeptide.

80. A method of controlling Lepidopteran insect comprising contacting said insects with an insecticidally-effective amount of a Bacillus thuringiensis Cry1C .delta.-endotoxin polypeptide having at least one amino acid mutation in the loop region between .alpha. helices 4 and 5 of domain 1 of said polypeptide, said polypeptide having improved insecticidal activity against Lepidopteran insects when compared to a native Cry1C .delta.-endotoxin polypeptide.

☐ 16. Document ID: US 5942602 A

L11: Entry 16 of 25 File: USPT Aug 24, 1999

DOCUMENT-IDENTIFIER: US 5942602 A
TITLE: Growth factor receptor antibodies

Brief Summary Text (5):
Various strategies have been employed to target the EGF receptor and/or his variants for tumor therapy. Monoclonal antibodies directed towards the extracellular domain of the EGF receptor have proven effective in the inhibition of tumor cell growth. The EGF receptor specific Mab 225 competes with EGF for binding to the EGF receptor thereby blocking ligand dependent receptor activation (Fan et al, 1993). Treatment with Mab 225 inhibits the growth of EGF receptor expressing tumor cells in vitro and in animal models in vivo (Masui et al., 1984; Ennis et al., 1989), and clinical studies with Mab 225 have recently been initiated. In an attempt to achieve more potent antitumoral effects recombinant fusion proteins have been constructed which contain the enzymatic domains of Pseudomonas exotoxin A (Chaudhary et al., 1987) or diphtheria toxin (Shaw et al., 1991), and employ the natural EGF receptor ligands TGF.alpha. or EGF for targeting to receptor overexpressing tumor cells. Due to the growth factor domain such toxins are able to activate the EGF receptor (Schmidt and Wels, 1996), which might facilitate rapid uptake by tumor cells, but could also be responsible for the significant cytotoxic activity displayed on cells expressing only moderate levels of the target receptor.

Brief Summary Text (6):
As an alternative to growth factors, single-chain immunotoxin (SCIT) fusion proteins can be used for the target cell specific delivery of therapeutic effector functions. SCIT fusion proteins are hybrid molecules that contain antibody variable regions (scFv) genetically fused to a binding defective toxin, most notably diphteria toxin (DT) and Pseudomonas exotoxin A (ETA). Both DT and PE halt protein synthesis by ADP-ribosylating elongation factor 2 upon entry to the cell cytosol resulting in cell death (Carroll and Collier, 1987). A recombinant single chain antibody-toxin consisting of a scFv domain derived from the antagonistic Mab 225 and truncated Pseudomonas exotoxin A was described recently (Wels et al., 1995).

Drawing Description Text (2):
FIGS. 1A-1B Schematic representation of the recombinant single-chain antibody-toxins scFv(14E1)-ETA and scFv(225)-ETA, and the growth factor toxin TGF.alpha.-ETA. The bacterially expressed scFv-ETA proteins consist of the scFv domains of the monoclonal antibodies 225 or 14E1 containing the heavy (V.sub.H) and light chain (V.sub.L) variable domains fused to amino acids 252 to 613 of Pseudomonas exotoxin A (ETA) representing the translocation domain II, domain Ib, and domain III which mediates the ADP ribosylation of the eukaryotic elongation factor 2. TGF.alpha.-ETA contains amino acids 1 to 50 of human TGF.alpha. as an EGF receptor specific binding domain. Included in the molecules are the synthetic cluster of 6 His residues N-terminal of ETA domain II facilitating the purification of the proteins via Ni.sup.2+ affinity chromatography (not shown).

Detailed Description Text (4):
Construction of scFv(14E1) and scFv(14E1)-ETA. 14E1 hybridoma cell mRNA was isolated using a Quick Prep RNA purification kit (Pharmacia Biotech, Brussels, Belgium). First strand cDNA synthesis was carried out according to the manufacturer's recommendations using a cDNA synthesis kit (Stratagene) with 100 ng mRNA and random primers. For amplification of the heavy (VH) and light chain (VL)

variable domains the first strand cDNA served as a template in a PCR as described (Wels el al., 1992). For amplification of the VH domain, 50 pmol each of the oligonucleotides 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ ID NO:9)and 5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC-3' (SEQ ID NO:10) were used, for amplification of the VL kappa domain, 50 pmol each of the oligonucleotides 5'-GCGACCTTGCACGCGTAGACATTGAGCTCACCCAGTCTCCA-3' (SEQ ID NO:11), and 5'-CGCTACAATAGCGGCCGCTACCGTCCGTTTGATTTCCAGCTTGGTGCC-3' (SEQ ID NO:12) or 5'-CGCTACATTAGCGGCCGCTACCGTCCGTTTCAGCTCCAGCTTGGTCCC-3' (SEQ ID NO:13) were used (M=A+C, (M=A+C, R=A+G, S=C+G, W=A+T, Y=C+T, K=G+T). Subsequently, the VH and VL PCR products products were reamplified using 50 pmol each of the oligonucleotide primers 5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ ID NO:14) and 5'-ATTATAAGCTTCAGGTSMARCTGCAGSAGTCWGG-3' (SEQ ID NO:15) (VH), or 5'-TTAGATCTCTAGAAKCTCGAGYTTKGTSC-3' (SEQ ID NO:16) and 5'-GACATTCAGCTGACCCAGWCTSC-3' (SEQ ID NO:17) (VL), respectively. PCR products were digested with HindIII and BstEII (VH), or with PvuII and XbaI (VL). Mab 14E1 VH cDNA was inserted into HindIII/BstEII digested plasmid pWW152 (Wels et al., 1992) which contains a sequence sequence encoding the 15 amino acid linker (GGGGS).sub.3. Subsequently, the 14E1 VL fragment was inserted 3' of the VH and linker sequences, resulting in the scFv (14E1I) encoding plasmid pWW152-14E1. For bacterial expression the scFv(14E1) sequence was isolated as a HindIII/XbaI fragment from pWW152 and inserted into HindIII/XbaI digested plasmid pSW50 (Wels et al., 1995). For the construction of the the scFv(14E1)-ETA toxin fusion the scFv(14E1) fragment was inserted into HindIII/XbaI digested plasmid pSW202 containing a truncated Pseudomonas ETA gene which lacks the original cell binding domain Ia of the toxin (Wels el al., 1995). The resulting plasmids pSW50-14E1 and pSW202-14E1 encode, respectively, the scFv (14E1) and scFv(14E1)-ETA proteins, fused at the N-terminus to the ompA signal sequence, the synthetic FLAG epitope, and a cluster of 6 His residues, under the control of an IPTG inducible tac promoter.

Detailed Description Text (5):
Expression and purification of scFv and scFv-ETA fusion proteins. Single colonies of E. coli CC118 carrying plasmids pSW50-14E1 or pSW50-225 (Wels et al., 1995) for the expression of EGF receptor specific scFv proteins, or plasmids pSW202-14E1 or pSW202-225 (Wels et al., 1995) for the expression of scFv-ETA fusion proteins were grown overnight at 37.degree. C. in LB medium supplemented with 0.6% glucose and 100 .mu.g/ml ampicillin. E. coli CC118 carrying plasmid pSW202-TGF.alpha. (Schmidt and Wels, 1996) were used for the expression of TGF.alpha.-ETA, a recombinant growth factor toxin which consists of amino acids 1 to 50 of human TGF.alpha. fused to truncated Pseudomonas ETA. The cultures were diluted 30-fold in the same medium, grown at 37.degree. C. to an OD.sub.550 of 0.7 and induced with 0.1 mM IPTG for 1 h at room temperature. Cells were harvested by centrifugation at 10,000 g for 10 min at 4.degree. C., the cell pellet from 11 of culture was resuspended in 15 ml PBS containing 6M guanidine hydrochloride, and lysed by sonication. Following incubation at room temperature for 30 min the lysate was clarified by centrifugation at 30,000 g for 30 min at 4.degree. C. The supernatant was diluted to 3M guanidine hydrochloride with PBS and recombinant proteins were purified via binding to Ni.sup.2+ -saturated chelating sepharose (Pharmacia Biotech). Specifically bound proteins were eluted with 3M guanidine hydrochloride, 250 mM imidazole in PBS. Fractions containing recombinant fusion proteins were pooled and dialysed twice against PBS, 400 mM L-arginine and PBS, respectively. Typical yield of purified proteins was 1 mg per 1 of original bacterial culture with a purity of approximately 70% determined by SDS-PAGE and Coomassie brilliant blue staining.

Detailed Description Text (13):
Construction and bacterial expression of scFv(14E1)-ETA. The 14E1 hybridoma producing a novel anti-EGF receptor monoclonal antibody (IgG. .kappa.) was derived by immunization of mice with human A43 1 epidermoid carcinoma cells and fusion of splenocytes following standard protocols. cDNAs encoding the heavy (VH) and light chain (VL) variable domains of the 14E1 Mab were derived from 14E1 hybridoma cell mRNA by reverse transcription and amplification using PCR. A single chain Fv gene

was created by connecting VH and VL sequences via a synthetic linker encoding the 15 15 amino acids (GGGGS).sub.3, and the scFv gene was fused to sequences encoding a truncated form of Pseudomonas aeruginosa exotoxin A (ETA) in the pSW202 vector as described (Wels et al., 1992 a; Wels et al., 1995). The resulting expression plasmid pSW202-14E1 encodes under the control of an IPTG inducible tac promoter a fusion protein consisting of the E. coli ompA signal peptide at the N-terminus, followed by the synthetic FLAG epitope, 6 His residues, the scFv(14E1), 6 His residues, and ETA amino acids 252 to 613. The structure of the scFv(14E1)-ETA gene product is schematically shown in FIG. 1A. The ETA portion of the molecule lacks the native cell binding domain Ia of the toxin but contains the translocation domain II which is required for processing of the toxin and release into the cytoplasm after internalization into target cells via the endosomal route, and the enzymatic domain III which catalyzes the ADP-ribosylation of eukaryotic elongation factor EF-2 thereby arresting cellular protein synthesis (Ogata et al., 1992).

CLAIMS:

9. A single chain polypeptide of claim 1, wherein the single chain polypeptide further comprises a third polypeptide segment comprising a cytotoxic effective portion of Pseudomonas exotoxin A.

18. A nucleic acid of claim 15, wherein the biologically-active component is a cytotoxic effective portion of Pseudomonas exotoxin A.

24. A double chain polypeptide having a binding affinity for an epidermal growth factor receptor, said polypeptide comprising:

(1) a first polypeptide segment comprising the binding portion of the heavy chain variable domain of monoclonal antibody 14E1; and

(2) a second polypeptide segment comprising the binding portion of the light chain variable domain of monoclonal antibody 14E1,

wherein the heavy chain variable domain is joined to the light chain variable domain by a covalent linkage extending from each of the domains.

25. A double chain polypeptide of claim 24, wherein the covalent linkage is a disulfide bond.

☐ 17. Document ID: US 5658871 A

L11: Entry 17 of 25 File: USPT Aug 19, 1997

DOCUMENT-IDENTIFIER: US 5658871 A
TITLE: Microbial lipase muteins and detergent compositions comprising same

Abstract Text (1):
Lipase enzymes including mutant lipase enzymes, e.g. from Pseudomonas species, are produced and modified by recombinant DNA technique. The enzymes are applicable in detergent and cleaning compositions, with advantages for example of improved stability to proteolytic digestion.

Brief Summary Text (2):

In particular the invention relates to the preparation and use of modified enzymes, especially modified lipases. Thus the invention as described below provides inter alia techniques for production of lipase, e.g. lipases of the Genus Pseudomonas, e.g. lipase from P. glumae (alias P. gladioli) and further provides genetically modified forms of such lipases and the use of such enzymes in detergent and cleaning compositions.

Brief Summary Text (8):
EP 0 331 376 (Amano) describes lipases and their Production by rDNA technique, and their use, including an aminoacid sequence of lipase from Pseudomonas cepacia.

Brief Summary Text (14):
Another attempt is described in WO 89/04361 (Novo), which concerns detergent compositions containing a lipase from Pseudomonas species and a protease from Fusarium or protease of subtilisin type which has been mutated in its amino acid sequence at positions 166, 169, or 222 in certain ways. It was reported that there was some reduction in the degree of attack upon the lipase by the particular proteases described.

Brief Summary Text (22):
For example, the invention provides mutant forms of lipases showing immunological cross-reactivity with antisera raised against lipases from Chromobacter viscosum var. lipolyticum NRRLB-3673, or against lipase from Alcaligenes PL-679, ATCC 31371 or FERM-P 3783 or against lipase from Pseudomonas fluorescens IAM 1057 and produced by an artificially modified microorganism containing a gene made by rDNA techniques which carries at least one mutation affecting the amino acid sequence of the lipase thereby to confer upon the lipase improved stability against attack by protease.

Brief Summary Text (25):
Correspondingly, artificially modified microorganisms according to the present invention can be produced on the basis of the following parent organisms among others, for example, from Escherichia coli, Pseudomonas aeruginosa, Ps. putida and modified strains of Ps. glumae in which the original gene for the lipase has been deleted, Bacillus subtilis, Saccharomyces cerevisiae and related species, Hansenula polymorpha and related species, and varieties of the genus Aspergillus. The parent organisms from which such artifically modified microorganisms are produced may be referred to as host cells or organisms. These host cells reflect a broad range of different micoorganisms, and other microorganisms not described in detail in the examples can also be used as host cells.

Brief Summary Text (40):
Still a further form of stabilisation of a bacterial lipase produced by rDNA technique, especially a lipase that shows immunological cross-reactivity with an antiserum raised against lipase from Chromobacter viscosum var lipolyticum NRRL B-3673, or against lipase from Alcaligenes PL-679, ATCC 31371 or FERM-P 3783, or against lipase from Pseudomonas fluorescens IAM 1057; can be achieved when the lipase (which can be but need not necessarily be of modified i.e. mutated aminoacid sequence) is expressed in an artificially modified heterologous and eukaryotic host microorganism, whereby the lipase is differently glycosylated than the lipase produced by the parent microorganism from which the gene or sequence originated that is expressed in the eukaryotic host.

Brief Summary Text (41):
The host organisms can be prokaryotic, for example a Gram (-) negative bacterium, for example a gram-negative bacterium selected from E. coli; Ps. aeruginosa; Ps. putida or Ps. glumae (alias Ps. gladioli); or a prokaryote selected from the genus Bacillus, Corynebacterium or Staphylococcus, and especially for example belonging to the species Pseudomonas putida and expressing a lipase gene derived from Ps. glumae (syn Ps. gladioli).

Brief Summary Text (43):
Examples of usable heterologous host cells for lipase prodcution include Escherichia coli, Pseudomonas aeruginosa, Ps. putida. Ps. glumae in which the original lipase gene has been deleted is a further suitable host. The preferred host systems for large scale production are Bacillus subilis, Saccharomyces cerevisiae and related species, Hansenula polymorpha and related species and members of the genus Aspergillus. Also suitable hosts for large scale production are Gram (-) negative bacteria specially selected and/or modified for efficient secretion of (mutant) lipases. As these host cells reflect a broad range of different microorganisms other microorganisms not described in detail in the examples can be used as well as host cells.

Brief Summary Text (44):
Within the preferred class of lipases the lipase produced by Pseudomonas glumae (formerly and more usually called Pseudomonas gladioli) is a preferred basis for the the processes and products of this invention. Neither the amino acid sequence nor the nucleotide sequence of the gene coding for the preferred lipase was previously known. The present inventors have isolated the gene coding for the preferred lipase of this bacterium as will be illustrated below.

Brief Summary Text (51):
(c) The gene coding for Pseudomonas glumae lipase contains two sites that can be glycolysated when this gene is transferred into an eukaryotic host. Glycosylation can have a stabilizing effect on the enzyme, particularly if the glycosylation occurs outside the active centre and the lipid binding region of the enzyme. On the other hand glycosylation can interfere with the lipid binding to the lipase.

Brief Summary Text (54):
A suitable and presently preferred example of a mutant lipase is based on lipase from Pseudomonas glumae with a His 154 Pro (H154P) mutation, which is believed to replace a site vulnerable to protease digestion in one of the loops of the tertiary structure of the lipase with a less vulnerable site.

Brief Summary Text (55):
According to the present invention it is found that modified (mutant) lipases from Pseudomonas or another of the preferred class of lipases, or lipases of modified or unmodified sequence expressed in heterologous producer organisms, e.g. with amino acid sequence modification(s) or changed glycosylation to increase the stability of the enzyme to protease digestion, are of value in detergent and cleaning compositions, especially for example in combination with proteases, e.g. proteases of the subtilisin type.

Brief Summary Text (56):
The invention thus provides, for example, a lipase having an aminoacid sequence substantially homologous with that of a bacterial lipase, e.g. that of Pseudomonas glumae, and produced by a heterologous and eukaryotic host microorganism on the basis of rDNA technique to introduce into said host microorganism a gene encoding the corresponding bacterial lipase or a mutant thereof, whereby said lipase is differently glycosylated than the lipase produced by the parent microorganism from which said gene originated.

Brief Summary Text (57):
According to a further aspect of the present invention it is found that modified (mutant) lipases from Pseudomonas or another of the preferred class of lipases with amino acid sequence modification(s) chosen to increase the net positive charge of the lipase and its pI, are also of value in detergent and cleaning compositions, especially for example in combination with proteases, e.g. proteases of the subtilisin type.

Brief Summary Text (61):

The table given below shows mutations carried by certain useful examples of mutant lipases according to the invention, based on the sequence of lipase from Pseudomonas glumae.

Brief Summary Text (93):
(a) a naturally occuring nucleotide sequence (e.g. that of FIG. 2) encoding the original amino acid sequence of the prelipase produced by Pseudomonas glumae (FIG. 2);

Brief Summary Text (95):
(c) nucleotide sequences encoding lipases that show positive immunological cross reaction against the antibodies raised against the lipase of Pseudomonas fluorescens IAM 1057 as described in EP 0 205 208 and EP 0 206 390;

Brief Summary Text (176):
Pseudomonas glumae strain PG1 as CBS 322.89

Brief Summary Text (177):
Pseudomonas glumae strain PG3 as CBS 323.89

Brief Summary Text (178):
Pseudomonas glumae strain PGT89 as CBS 262.90

Brief Summary Paragraph Table (1):
TABLE _____ Mutant lipases based on Pseudomonas glumae lipase sequence: Strain (label): Mutation
_____ PGL4 V150A PGL5 V150S PGL6 V150D PGL7 V150K PGL8 D159E PGL24 H154P PGL31 S153P PGL27 S153R PGL33 H154R PGL39 D157R PGL32 S153G PGL34 H154G PGL37 S153P + H154P PGL55 S152P + H154P PGL56 V150P + H154P PGL58 V150P + S152P + H154P PGL36 S153R + H154R PGL38 S153G + H154G PGL57 S151R + H154P PGL35 S153G + H154P PGL59 S152* + S153* + H154P PGL40 S152A + *152aL + *153aG + *154aP (sequence SSH at positions 152-4 becomes ALSGHP, with 3 net insertions) PGL41 S152A + *152aL + *153aG + *154aP + N155R + T156L + D157P + D159N (sequence SSHNTDQD at positions 152-9 becomes ALSGHPRLPQN with 3 net insertions) PGL42 N48S PGL43 N238S PGL44 *155aG PGL45 D157T PGL46 N48S + N238S PGL12 H15A PGL13 T109D PGL14 T110K PGL16 R8D PGL17 R8Q PGL18 R61P PGL19 K70Q PGL20 A74S PGL21 S87A PGL22 R94D PGL23 R49Q PGL28 D55A PGL29 D56A PGL30 D263E PGL60 D121E PGL61 D287E PGL62 H285A PGL63 M254I _____

Drawing Description Text (3):
FIG. 2: Complete nucleotide sequence (1074 bp) and aminoacid sequence of the lipase gene from Pseudomonas glumae, with amino terminal encoded by nucleotides 118 et seq. seq.

Detailed Description Text (335):
E. Transformation of Pseudomonas putida.

Detailed Description Text (360):
In order to identify useful mutations for introduction into the aminoacid sequence of lipase enzymes, the site of primary cleavage of the lipase polypeptide under the action of subtilisin can be determined as follows. The details refer primarily to work carried out with the lipase of Pseudomonas glumae, and can be modified for other lipases.

Detailed Description Text (365):
Corresponding treatment of lipase from Pseudomonas cepacia (of sequence published in in EP 0 331 376 (Amano)) showed a primary subtilitic cleavage site between Ser152 and Ser153 of the mature lipase.

Detailed Description Text (366):

Mutations in lipase of Pseudomonas glumae to confer improved resistance to subtilisin degradation, and constructed by the methods described above, include the following examples: mutant PGL24, carrying mutation H154P, and mutant PGL6, carrying mutation V150D. In both these cases the mutant lipase enzyme has been tested and shown to have significantly reduced susceptibility to proteolytic attack. Correponding useful mutations in lipase of Pseudomonas cepacia include for example S153P.

CLAIMS:

1. A substantially pure Pseudomonas lipase mutein produced from a microorganism by recombinant DNA technique, and maintaining an amino acid substitution at a position within its amino acid sequence homologous to position 150 in the corresponding sequence of the mature, secreted lipase of Pseudomonas glumae, wherein said mutation confers improved stability against attack by a subtilisin protease.

2. A Pseudomonas lipase mutein as claimed in claim 1, which shows immunological cross-reactivity with an antiserum raised against lipase from Chromobacter viscosum var lipolyticum NRRLB-3673, or against lipase from Alcaligenes PL-679, ATCC 31371 or FERM-P 3783, or against lipase from Pseudomonas fluorescens IAM 1057.

3. A Pseudomonas lipase mutein as claimed in claim 1, in which the aminoacid sequences have been modified by recombinant DNA technique in such a way that loop structures of the lipase are stabilized against physical or chemical denaturation or enzymic cleavage.

4. A Pseudomonas lipase mutein as claimed in claim 1, with aminoacid sequence modification(s) chosen to improve stability of lipase against attack by subtilisin protease, by (i) modifying the sequence at a subtilisin cleavage site in the sequence within five residues on either side of a bond susceptible in the parent enzyme to subtilisin cleavage by deletion of one to three aminoacid residues forming part of such a site as it existed before mutation, or (ii) by introducing by insertion or substitution at least one basic aminoacid residue, or at least one proline residue, at such a cleavage site, or (iii) modifying the electrostatic potential at such a site by introduction of positively charged aminoacids or removal of negatively charged aminoacids at such a site.

5. A Pseudomonas lipase mutein as claimed in claim 4, wherein (i) the aminoacid residue that would become a new N-terminal upon cleavage of the bond susceptible in the parent enzyme to subtilisin cleavage, is replaced by proline; and/or (ii) an aminoacid residue two or four positions from the susceptible bond is replaced by a charged or more polar aminoacid residue.

7. A Pseudomonas lipase according to claim 2, and in which part of the nucleotide sequence has been replaced by a substantially corresponding part of a nucleotide sequence encoding another lipase, immunologically related to the first.

8. A substantially pure Pseudomonas lipase mutein as claimed in any of claims 1, 2, 3, 4, 5, 6 or 7, with one or more of the following aminoacid sequence modification (s) relative to the sequence of Ps. glumae lipase or a homologue thereof:

V150A; V150S; V150D; V150K; V150P+H154P; V150P+S152P+H154P.

9. A substantially pure Pseudomonas lipase mutein according to claim 1, having an amino acid sequence substantially as shown in FIG. 2 or a functional equivalent thereof, and derived from an artificially modified microorganism containing a modified gene substantially corresponding to a prepro-lipase sequence also as shown in FIG. 2 or a functional equivalent thereof.

10. A detergent composition comprising a Pseudomonas lipase mutein or protein

according to claim 1, and optionally a subtilisin protease enzyme, wherein the remainder of the detergent composition is either:

(a) formulated as a detergent powder containing phosphate builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, perborate or peracid peracid bleach precursor, amino-containing bleach activator, silicate or other structurant, alkali to adjust to desired pH in use, and neutral inorganic salt; or

(b) formulated as a detergent powder containing zeolite builder, anionic surfactant, surfactant, nonionic surfactant, acrylic or equivalent polymer, perborate or peracid peracid bleach precursor, amino-containing bleach activator, silicate or other structurant, alkali to adjust to desired pH in use, and neutral inorganic salt; or

(c) formulated as an aqueous detergent liquid comprising anionic surfactant, nonionic surfactant, humectant, organic acid or other builder, caustic alkali, with a pH adjusted to a value between 9 and 10; or

(d) formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant consisting essentially of linear alkoxylated primary alcohol, triacetin, sodium triphosphate, caustic alkali, perborate monohydrate bleach precursor, and tertiary amino bleach activator, with a pH adjusted to a value between about 9 and 10; or

(e) formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and a mixture of nonionic nonionic surfactants with respective alkoxylation degrees about 7 and about 3, low or substantially zero neutral inorganic salt, phosphate builder, perborate or peracid bleach precursor, tertiary amine bleach activator, sodium silicate, and minors and moisture; or

(f) formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and a mixture of nonionic nonionic surfactants with respective alkoxylation degrees about 7 and about 3, low or substantially zero neutral inorganic salt, zeolite builder, perborate or peracid bleach precursor, tertiary amino bleach activator, sodium silicate, and minors and moisture; or

(g) formulated as a detergent powder containing anionic surfactant, nonionic surfactant, acrylic polymer, fatty acid soap, sodium carbonate, sodium sulphate, clay particles, perborate or peracid bleach precursor, tertiary amine bleach activator, sodium silicate, and minors and moisture; or

(h) formulated as a soap or synthetic detergent bar containing either soap based on pan-saponified mixture of tallow and coconut oil, neutralized with orthophosphoric acid, or C6-C16 alkylbenzenesulphonate, sodium tripolyphosphate, calcium and sodium carbonate and carboxymethylcellulose, mixed with protease, also mixed with sodium formate, borax, propylene glycol and sodium sulphate, and then plodded on a soap production line.

☐ 18. Document ID: US 5648237 A

L11: Entry 18 of 25 File: USPT Jul 15, 1997

DOCUMENT-IDENTIFIER: US 5648237 A
TITLE: Expression of functional antibody fragments

Detailed Description Text (53):
Suitable host cells for expressing Fab are microbial cells such as yeast, fungi, and prokaryotes. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, E. coli, Bacilli such as B. subtilis, Pseudomonas species such as P. aeruginosa, Salmonella typhimurium, or Serratia marcescans. One preferred E. coli cloning host is E. coli 294 (ATCC 31,446), although other strains such as E. coli B, E. coli .sub.X 1776 (ATCC 31,537), E. coli RV308(ATCC 31,608) and E. coli W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Detailed Description Text (65):
The Fab-containing polypeptides also may comprise an immunotoxin. For example, the Fab heavy chain is optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. U.S. patent application Ser. No. 07/350,895 illustrates methods for making and using immunotoxins for the treatment of HIV infection, and its teachings are specifically incorporated by reference herein. Alternatively, the toxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAR, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. In another embodiment, the antibodies are conjugated to nonpeptidyl drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCI, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis- (p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab antibody fragment.

CLAIMS:

12. The method of claim 11, wherein said covalent bond is a disulfide bond.

37. The method of claim 19 wherein the signal sequence comprises the heat-stable enterotoxin II signal sequence.

38. The method of claim 1 wherein the microbial host cell in step (a) is transformed with a vector comprising said nucleic acid operably linked to control sequences recognized by the host cell such that the Fab' is secreted to the periplasmic space of the host cell.

41. The method of claim 11 wherein the microbial host cell in step (a) is transformed with a vector comprising the nucleic acid encoding the first Fab' operably linked to control sequences recognized by the host cell such that the first first Fab' is secreted to the periplasmic space of the host cell, and wherein the microbial host cell of step (b) is transformed with a vector comprising the nucleic acid encoding the second Fab' operably linked to control sequences recognized by the

the host cell such that the second Fab' is secreted to the periplasmic space of the host cell.

45. The method of claim 32 wherein the protective group is selected from the group consisting of TNB, p-methoxybenzyl and pyridine disulfide.

☐ 19. Document ID: US 5601825 A

L11: Entry 19 of 25 File: USPT Feb 11, 1997

DOCUMENT-IDENTIFIER: US 5601825 A
TITLE: Therapeutic conjugates of toxins and drugs

Brief Summary Text (56):
Pseudomonas aeruginosa

Brief Summary Text (146):
Methods for treating toxins and, in particular, modified Psuedomonas exotoxins, are disclosed in Batkra et al., Proc. Natl. Acad. Sci. USA, Vol. 86, pp. 8545-8549, 1989; Seetharam et al., The Journal of Biol. Chem., Vol. 266, No. 26, pp. 17376-17381, 1991; and Pastan et al., U.S. Pat. No. 4,892,827, all incorporated herein by reference. A preferred modified Pseudomonas exotoxin comprises ADP ribosylating activate, an ability to translocate across a cell membrane and devoid of a functional receptor binder region Ia of the native toxin. One such modified Pseudomonas exotoxin is devoid of amino acids 1-252 and 365-380 of native Pseudomonas exotoxin and contains a -KDEL mutation instead of -REDLK at the carboxyl carboxyl terminus.

Detailed Description Text (8):
D. A modified Pseudomonas Exotoxin (mPE), lacking the Ia binding domain, is expressed in E. Coli, extracted from the periplasm, and purified by ion-exchange chromatography; mPE is activated by treatment with SMCC (AmPE).

CLAIMS:

1. A method of treating patients having a tumor or a disease caused by an infectious agent, said method comprising the step of administering a hypoimmunogenic immunoconjugate that comprises an antibody Fab or Fab' fragment that specifically binds to a tumor-associated antigen of a targeted tumor cell or an antigen associated with a protozoan, said tumor-associated antigen being on a lymphoma, carcinoma, sarcoma, leukemia or myeloma cell, wherein said antibody fragment is conjugated through a first thiol-binding linker to a drug or modified toxin devoid of a functional receptor-binding domain, and wherein said antibody fragment is further conjugated through at least a second thiol-binding linker to at least one polysaccharide or polyol group, wherein the antibody thiol groups linked to said linkers are derived from reduction of heavy chain disulfide bonds, and wherein said antigen internalizes said conjugate into the cytoplasm of the cells of said tumor, into the cytoplasm of cells containing said protozoan, or into said protozoan itself.

6. The method of claim 5, wherein said toxin is a modified Pseudomonas exotoxin.

10. A method of cancer therapy, said method comprising the step of parenterally injecting into a human subject having a cancer which produces or is associated with an antigen, a cytotoxic amount of a Fab or Fab' antibody fragment that specifically binds to said antigen, wherein said antibody fragment is conjugated through a first thiol-binding linker to a drug or modified toxin devoid of a functional receptor-binding domain, and wherein said antibody fragment is further conjugated through at least a second thiol-binding linker to at least one polysaccharide or polyol group, wherein the antibody thiol groups linked to said linkers are derived from reduction of heavy chain disulfide bonds, wherein said antigen internalizes said conjugate into the cytoplasm of the cells of said cancer, and wherein said cancer is selected from the group consisting of lymphoma, carcinoma, sarcoma, leukemia and myeloma.

12. The method of claim 11, wherein said toxin is a modified Pseudomonas exotoxin.

☐ 20. Document ID: US 5571698 A

L11: Entry 20 of 25 File: USPT Nov 5, 1996

DOCUMENT-IDENTIFIER: US 5571698 A
**** See image for Certificate of Correction ****
TITLE: Directed evolution of novel binding proteins

Detailed Description Text (97):
If the target is a small molecule, such as a steroid, a preferred embodiment of the IPBD is a protein of about 80-200 residues, such as ribonuclease from Bos taurus (124 residues), ribonuclease from Aspergillus oruzae (104 residues), hen egg white lysozyme from Gallus gallus (129 residues), azurin from Pseudomonas aerugenosa (128 residues), or T4 lysozyme (164 residues), because such proteins have clefts and grooves into which the small target molecules can fit. The Brookhaven Protein Data Bank contains 3D structures for all of the proteins listed. Genes encoding proteins as large as T4 lysozyme can be manipulated by standard techniques for the purposes of this invention.

Detailed Description Text (331):
Viruses are preferred over bacterial cells and spores (cp. LUIT85 and references cited therein). The virus is preferably a DNA virus with a genome size of 2 kb to 10 kb base pairs, such as (but not limited to) the filamentous (Ff) phage M13, fd, and f1 (inter alia see RASC86, BOEK80, BOEK82, DAYL88, GRAY81b, KUHN88, LOPE85, WEBS85, MARV75, MARV80, MOSE82, CRIS84, SMIT88a, SMIT88b); the IncN specific phage Ike and If1 (NAKA81, PEET85, PEET87, THOM83, THOM88a); IncP-specific Pseudomonas aeruginosa phage Pf1 (THOM83, THOM88a) and Pf3 (LUIT83, LUIT85, LUTI87, THOM88a); and the Xanthomonas oryzae phage Xf (THOM83, THOM88a). Filamentous phage are especially preferred.

Detailed Description Text (360):
Similar constructions could be made with other filamentous phage. Pf3 is a well known filamentous phage that infects Pseudomonas aerugenosa cells that harbor an IncP-1 plasmid. The entire genome has been sequenced (LUIT85) and the genetic signals involved in replication and assembly are known (LUIT87). The major coat protein of PF3 is unusual in having no signal peptide to direct its secretion. The

sequence has charged residues ASP.sub.7, ARG.sub.37, LYS.sub.40, and PHE.sub.44 - COO.sup.- which is consistent with the amino terminus being exposed. Thus, to cause an IPBD to appear on the surface of Pf3, we construct a tripartite gene comprising:

Detailed Description Text (397):
Among bacterial cells, the preferred genetic packages are Salmonella typhimurium, Bacillus subtilis, Pseudomonas aeruginosa, Vibrio cholerae, Klebsiella pneumonia, Neisseria gonorrhoeae, Neisseria meningitidis, Bacteroides nodosus, Moraxella bovis, and especially Escherichia coli. The potential binding mini-protein may be expressed as an insert in a chimeric bacterial outer surface protein (OSP). All bacteria exhibit proteins on their outer surfaces. Works on the localization of OSPs and the methods of determining their structure include: CALA90, HEIJ90, EHRM90, BENZ88a, BENZ88b, MANO88, BAKE87, RAND87, HANC87, HENR87, NAKA86b, MANO86, SILH85, TOMM85, NIKA84, LUGT83, and BECK83.

Detailed Description Text (410):
Other bacterial outer surface proteins, such as OmpA, OmpC, OmpF, PhoE, and pilin, may be used in place of LamB and its homologues. OmpA is of particular interest because it is very abundant and because homologues are known in a wide variety of gram-negative bacterial species. Baker et al. (BAKE87) review assembly of proteins into the outer membrane of E. coli and cite a topological model of OmpA (VOGE86) that predicts that residues 19-32, 62-73, 105-118, and 147-158 are exposed on the cell surface. Insertion of a ipbd encoding fragment at about codon 111 or at about codon 152 is likely to cause the IPBD to be displayed on the cell surface. Concerning OmpA, see also MACI88 and MANO88. Porin Protein F of Pseudomonas aeruginosa has been cloned and has sequence homology to OmpA of E. coli (DUCH88). Although this homology is not sufficient to allow prediction of surface-exposed residues on Porin Protein F, the methods used to determine the topological model of OmpA may be applied to Porin Protein F. Works related to use of OmpA as an OSP include BECK80 and MACI88.

Detailed Description Text (620):
10) Pseudomonas putida cytochrome P450.sub.CAM

Detailed Description Text (642):
5) Pseudomonas aeruginosa hemolysin

Detailed Description Text (1424):
DUCH88: Duchene, M, A Schweized, F Lottspeich, G Krauss, M Marget, K Vogel, B-U von Specht, and H Domdey, "Sequence and Transcriptional Start Site of the Pseudomonas aeruginosa Outer Membrane Porin Protein F Gene", J Bacteriol (1987),170:155-162.

Detailed Description Text (1566):
LUIT83: Luiten, R G M, J G G Schoenmakers, and R N H Konings, "The major coat protein gene of the filamentous Pseudomonas aeruginosa phage Pf3: absence of an N-terminal leader signal sequence", Nucleic Acids Research (1983), 11(22)8073-85.

Detailed Description Text (1567):
LUIT85: Luiten, R G M, D G Putterman, J G G Schoenmakers, R N H Konings, and L A Day, "Nucleotide Sequence of the Genome of Pf3, an IncP-1 Plasmid-Specific Filamentous Bacteriophage of Pseudomonas aeruginosa", J Virology, (1985), 56(1) 268-268-276.

Detailed Description Text (1732):
TRIA88: Trias, J, E Y Rosenberg, and H Nikaido, "Specificity of the glucose channel formed by protein D1 of Pseudomonas aeruginosa", Biochim Biophys Acta (1988), 938:493-496.

CLAIMS:

7. The method of claim 3 wherein the initially chosen parental potential binding domain is selected from the group consisting of (a) binding domains of bovine pancreatic trypsin inhibitor, crambin, Cucurbita maxima trypsin inhibitor III, heat-heat-stable enterotoxin of Excherichia coli, .alpha. Conotoxin GI, .mu.Conotoxin GIII, .omega. Conotoxin GIV, apamin, charybdotoxin, secretory leukocyte protease inhibitor, cystatin, eglin, barley protease inhibitor, ovomucoid, T4 lysozyme, hen egg white lysozyme, ribonuclease, azurin, tumor necrosis factor, and CD4, and (b) domains at least substantially homologous with any of the foregoing domains which have a melting point of at least 50.degree. C.

11. The method of claim 3 wherein the initially chosen parental binding domain contains no more than 30 residues and at least 2 disulfides.

12. The method of claim 3 wherein the initially chosen parental binding domain contains no more than 60 residues and at least 3 disulfides.

13. The method of claim 3 wherein the initially chosen parental binding domain contains no more than 80 residues and at least 4 disulfides.

22. The method of claim 21 wherein the bacterial cell is selected from the group consisting of strains of Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumonia, Neisseria gonorrhoeae, and Bacillus subtilis.

25. The method of claim 23 wherein the chimeric potential binding protein substantially corresponds to the first 153 amino acids of Lamb fused in frame to the potential binding domain.

35. The method of claim 34 wherein the crosslink is a disulfide bond and the the amino acids at the first and second, amino acid positions are cysteines.

36. The method of claim 35 in which the mini-protein domain has a single disulfide bond and the span of the bond is not more than nine amino acid residues.

37. The method of claim 35 wherein the mini-protein domain has a disulfide bond which bridges a sequence of amino acids which under affinity separation conditions collectively assume a hairpin supersecondary structure.

39. The method of claim 35 wherein the mini-protein domain comprises a plurality of intrachain disulfide bonds.

40. The method of claim 39 wherein the mini-protein domain has two disulfide bonds having a connectivity pattern of 1-3 , 2-4.

42. The method of claim 39 wherein the mini-protein domain has three disulfide bonds having a connectivity pattern of 1-4, 2-5, 3-6.

62. The method of claim 59 wherein the second binding protein is produced by recombinant DNA techniques.

75. The method of claim 73 wherein the crosslink is a disulfide bond and the amino acids at said first and second positions are cysteines.

☐ 21. Document ID: US 5541297 A

L11: Entry 21 of 25 File: USPT Jul 30, 1996

DOCUMENT-IDENTIFIER: US 5541297 A
TITLE: Therapeutic conjugates of toxins and drugs

Detailed Description Text (25):
Pseudomonas aeruginosa

Detailed Description Text (115):
Methods for treating toxins and, in particular, modified Psuedomonas exotoxins, are disclosed in Batkra et al., Proc. Natl. Acad. Sci. USA, Vol. 86, pp. 8545-8549, 1989; Seetharam et al., The Journal of Biol. Chem., Vol 266, no. 26, pp. 17376-17381, 1991; and Pastan et al., U.S. Pat. No. 4,892,827, all incorporated herein by reference. A preferred modified Pseudomonas exotoxin comprises ADP ribosylating activate, an ability to translocate across a cell membrane and devoid of a functional receptor binder region Ia of the native toxin. One such modified Pseudomonas exotoxin is devoid of amino acids 1-252 and 365-380 of native Pseudomonas exotoxin and contains a -KDEL mutation instead of -REDLK at the carboxyl carboxyl terminus.

Detailed Description Text (128):
D. A modified Pseudomonas Exotoxin (mPE), lacking the Ia binding domain, is expressed in E. Coli, extracted from the periplasm, and purified by ion-exchange chromatography; mPE is activated by treatment with SMCC (AmPE).

CLAIMS:

1. A hypoimmunogenic immunoconjugate, which comprises an antibody Fab or Fab' fragment that specifically binds to a tumor-associated antigen of a targeted tumor cell or an antigen associated with a protozoan, said tumor associated antigen being on a lymphoma, carcinoma, sarcoma, leukemia or myeloma cell, wherein said antibody fragment is conjugated through a first thiol-binding linker to a drug or modified toxin devoid of a functional receptor-binding domain, and further conjugated through at least a second thiol-binding linker to at least one polysaccharide or polyol group, wherein the antibody thiol groups linked to said linkers are derived from reduction of heavy chain disulfide bonds; and wherein said antigen internalizes internalizes said conjugate into the cytoplasm of said targeted cell or microbe.

4. The immunoconjugate of claim 3, wherein said toxin is a modified Pseudomonas exotoxin.

8. A method of producing a hypoimmunogenic immunoconjugate, comprising the steps of:

(a) partially reducing an intact antibody that specifically binds to a tumor-associated antigen of a targeted tumor cell or an antigen associated with a protozoan, said tumor-associated antigen being on a lymphoma, carcinoma, sarcoma, leukemia or myeloma cell, wherein said reduction is effected with a reducing agent for cleaving disulfide groups, in an amount sufficient to generate a plurality of proximal free sulfhydryl groups but insufficient to render immunologically inactive said antibody or to completely cleave the antibody heavy chain, and recovering partially reduced antibody;

(b) enzymatically cleaving said partially reduced antibody with pepsin or papain to generate a F(ab').sub.2 or F(ab).sub.2 fragment, and recovering said fragment; and

either

(c) reacting said F(ab').sub.2 or F(ab).sub.2 fragment with at least one polysaccharide or polyol group coupled to a thiol-reactive linker, to conjugate said said polysaccharide or polyol group to said fragment, and recovering the resultant conjugate;

(d) cleaving the remaining disulfide groups linking the heavy chains of the product of step (c) with a reducing agent for cleaving disulfide groups, and recovering the resultant polysaccharide- or polyol-conjugated Fab' or Fab fragment having at least one free sulfhydryl group; and

(e) reacting the product of step (d) with a drug or modified toxin devoid of a functional receptor-binding domain and coupled to a thiol-reactive linker, and recovering the resultant hypoimmunogenic immunoconjugate, or

(c') reacting said F(ab').sub.2 or F(ab).sub.2 fragment with a drug or modified toxin devoid of a functional receptor-binding domain and coupled to a thiol-reactive linker, and recovering the resultant conjugate;

(d') cleaving the remaining disulfide groups linking the heavy chains of the product product of step (c') with a reducing agent for cleaving disulfide groups, and recovering the resultant drug- or modified toxin-conjugated Fab' or Fab fragment having at least one free sulfhydryl group; and

(e') reacting the product of step (d') with at least one polysaccharide or polyol group coupled to a thiol-reactive linker, to conjugate said polysaccharide or polyol group to said fragment, and recovering the resultant hypoimmunogenic immunoconjugate.

☐ 22. Document ID: US 5403484 A

L11: Entry 22 of 25 File: USPT Apr 4, 1995

DOCUMENT-IDENTIFIER: US 5403484 A
TITLE: Viruses expressing chimeric binding proteins

Detailed Description Text (95):
If the target is a small molecule, such as asteroid, a preferred embodiment of the .IPBD is a protein of about 80-200 residues, such as ribonuclease from BOS taurus (124 residues), ribonuclease from Aspergillus oruzae (104 residues), hen egg white lysozyme from Gallus gallus (129 residues), azurin from Pseudomonas aerugenosa (128 residues), or T4 lysozyme (164 residues), because such proteins have clefts and grooves into which the small target molecules can fit. The Brookhaven Protein Data Bank contains 3D structures for all of the proteins listed. Genes encoding proteins as large as T4 lysozyme can be manipulated by standard techniques for the purposes of this invention.

Detailed Description Text (330):
Viruses are preferred over bacterial cells and spores (cp. LUIT85 and references cited therein). The virus is preferably a DNA virus with a genome size of 2 kb to

10 kb base pairs, such as (but not limited to) the filamentous (Ff) phage M13, fd, and f1 (inter alia see RASC86, BOEK80, BOEK82, DAYL88, GRAY81b, KUHN88, LOPE85, WEBS85, MARV75, MARV80, MOSE82, CRIS84, SMIT88a, SMIT88b) ; the IncN specific phage Ike and If1 (NAKA81, PEET85, PEET87, THOM83, THOM88a); IncP-specific Pseudomonas aeruginosa phage Pf1 (THOM83, THOM88a) and Pf3 (LUIT83, LUIT85, LUTI87, THOM88a); and the Xanthomonas oryzae phage Xf (THOM83, THOM88a). Filamentous phage are especially preferred.

Detailed Description Text (359):
Similar constructions could be made with other filamentous phage. Pf3 is a well known filamentous phage that infects Pseudomonas aerugenosa cells that harbor an IncP-1 plasmid. The entire genome has been sequenced (LUIT85) and the genetic signals involved in replication and assembly are known (LUIT87). The major coat protein of PF3 is unusual in having no signal peptide to direct its secretion. The sequence has charged residues ASP.sub.7, ARG.sub.37, LYS.sub.40, and PHE.sub.44 - COO.sup.- which is consistent with the amino terminus being exposed. Thus, to cause an IPBD to appear on the surface of Pf3, we construct a tripartite gene comprising:

Detailed Description Text (396):
Among bacterial cells, the preferred genetic packages are Salmonella typhimurium, Bacillus subtilis, Pseudomonas aeruginosa, Vibrio cholerae, Klebsiella pneumonia, Neisseria gonorrhoeae, Neisseria meningitidis, Bacteroides nodosus, Moraxella bovis, and especially Escherichia coli. The potential binding mini-protein may be expressed as an insert in a chimeric bacterial outer surface protein (OSP). All bacteria exhibit proteins on their outer surfaces. Works on the localization of OSPs and the methods of determining their structure include: CALA90, HEIJ90, EHRM90, BENZ88a, BENZ88b, MANO88, BAKE87, RAND87, HANC87, HENR87, NAKA86b, MANO86, SILH85, TOMM85, NIKA84, LUGT83, and BECK83.

Detailed Description Text (409):
Other bacterial outer surface proteins, such as OmpA, OmpC, OmpF, PhoE, and pilin, may be used in place of LamB and its homologues. OmpA is of particular interest because it is very abundant and because homologues are known in a wide variety of gram-negative bacterial species. Baker et al. (BAKE87) review assembly of proteins into the outer membrane of E. coli and cite a topological model of OmpA (VOGE86) that predicts that residues 19-32, 62-73, 105-118, and 147-158 are exposed on the cell surface. Insertion of a ipbd encoding fragment at about codon 111 or at about codon 152 is likely to cause the IPBD to be displayed on the cell surface. Concerning OmpA, see also MACI88 and MANO88. Porin Protein F of Pseudomonas aeruginosa has been cloned and has sequence homology to OmpA of E. coli (DUCH88). Although this homology is not sufficient to allow prediction of surface-exposed residues on Porin Protein F, the methods used to determine the topological model of OmpA may be applied to Porin Protein F. Works related to use of OmpA as an OSP include BECK80 and MACI88.

Detailed Description Text (619):
10) Pseudomonas putida cytochrome P450.sub.CAM

Detailed Description Text (641):
5) Pseudomonas aeruginosa hemolysin

CLAIMS:

1. A virus bearing on its outer surface a chimeric binding protein, said protein comprising (i) a proteinaceous binding domain, other than a single chain antibody, which is sufficiently stable in structure to have a melting point of at least 40.degree. C., and which binds to a target:, other than the variable domain of an antibody, sufficiently strongly so that the dissociation constant of the binding domain: target complex is less than 10.sup.-6 moles/liter, and (ii) at least a

functional portion of a coat protein of said virus, said portion acting, when the chimeric protein is produced in a suitable host cell, to cause the display of the chimeric binding protein or a processed form thereof on the outer surface of the virus, said binding domain being capable of binding to a target material which said coat protein does not preferentially bind, said binding domain being foreign to the native coat proteins of said virus.

7. The virus of claim 1 wherein the proteinaceous binding domain features at least two disulfides.

8. The virus of claim 1 wherein the proteinaceous binding domain has a single disulfide bond and the span of the bond is not more than nine amino acids.

9. The virus of claim 1 wherein the proteinaceous binding domain contains no more than 30 residues and at least 2 disulfides.

10. The virus of claim 1 wherein the proteinaceous binding domain contains no more than 60 residues and at least 3 disulfides.

11. The virus of claim 1 wherein the proteinaceous binding domain contains no more than 80 residues and at least 4 disulfides.

22. The virus of claim 1 wherein the binding domain is at least substantially homologous with a binding domain selected from the group consisting of the binding domains of bovine pancreatic trypsin inhibitor, crambin, Cucurbita maxima trypsin inhibitor III, heat stable enterotoxin of Escherichia coli, .alpha. Conotoxin GI, .lambda. Conotroxin GIII, .omega. Conotoxin GIV, apamin, charybdtoxin, secretory leukocyte protease inhibitor, cystatin, eglin, barley protease inhibitor, ovomucoid, T4 lysozyme, hen egg white lysozyme, ribonuclease, azurin, tumor necrosis factor, and CD4.

26. A chimeric binding protein comprising (i) a proteinaceous binding domain, other than a single chain antibody, which is sufficiently stable in structure to have a melting point of at least 40.degree. C., and which binds to a target, other than the variable domain of an antibody, sufficiently strongly so that the disassociation constant of the binding domain: target complex is less that 10.sup.-6 moles/liter, and (ii) at least a functional portion of a coat protein of a virus, said portion acting, when the chimeric protein is produced in a suitable host cell, to cause the display of the chimeric binding protein or a processed form thereof on the outer surface of the virus, said binding domain being capable of binding to a target material which said coat protein does not preferentially bind, said binding domain being foreign to the native coat proteins of said virus.

32. The protein of claim 26 wherein the binding domain features at least two disulfides.

33. The protein of claim 26 wherein the proteinaceous binding domain has a single disulfide bond and the span of the bond is not more than nine amino acids.

34. The protein of claim 26 wherein the proteinaceous binding domain contains no more than 30 residues and at least 2 disulfides.

35. The protein of claim 26 wherein the proteinaceous binding domain contains no more than 60 residues and at least 3 disulfides.

36. The protein of claim 28 wherein the proteinaceous binding domain contains no more than 80 residues and at least 4 disulfides.

44. A fusion protein comprising (a) a carrier protein moiety essentially corresponding to a mature gene III protein of a filamentous phage, said carrier

protein moiety acting, when the fusion protein is produced in a suitable host cell infected by the phage, to cause the display of the fusion protein or a processed form thereof on the surface of the phage, and (b) a foreign peptide or protein coupled to the amino terminal of said carrier protein moiety.

45. A recombinant filamentous phage bearing a fusion protein according to claim 44, upon its outer surface, said carrier protein moiety being integrated into the coat of the phage, said foreign peptide or protein being capable of binding specifically to a target which said phage does not specifically bind and being of an amino acid sequence foreign to the coat proteins native to said phage.

46. A library of recombinant phage according to claim 45, said library displaying a plurality of different foreign peptides or proteins.

47. A fusion protein comprising (a) at least a functional portion of a mature gene VIII protein of a filamentous phage, said portion acting, when the fusion protein is produced in a suitable host cell infected by the phage to cause the display of the fusion protein or a processed form thereof on the surface of the phage, and (b) a foreign peptide or protein coupled to said functional portion of said mature gene VIII protein.

48. A recombinant filamentous phage bearing a fusion protein according to claim 47, upon its outer surface, said functional portion of the gene VIII protein being integrated into the coat of the phage, said foreign peptide or protein being capable of binding specifically to a target which said phage does not specifically bind and being of an amino acid sequence foreign to the coat proteins native to said phage.

49. A library of recombinant phage according to claim 48, said library displaying a plurality of different foreign peptides or proteins.

☐ 23. Document ID: US 5223409 A

L11: Entry 23 of 25 File: USPT Jun 29, 1993

DOCUMENT-IDENTIFIER: US 5223409 A
TITLE: Directed evolution of novel binding proteins

Detailed Description Text (97):
If the target is a small molecule, such as a steroid, a preferred embodiment of the IPBD is a protein of about 80-200 residues, such as ribonuclease from Bos taurus (124 residues), ribonuclease from Asperoillus oruzae (104 residues), hen egg white lysozyme from Gallus gallus (129 residues), azurin from Pseudomonas aerugenosa (128 residues), or T4 lysozyme (164 residues), because such proteins have clefts and grooves into which the small target molecules can fit. The Brookhaven Protein Data Bank contains 3D structures for all of the proteins listed. Genes encoding proteins as large as T4 lysozyme can be manipulated by standard techniques for the purposes of this invention.

Detailed Description Text (322):
Viruses are preferred over bacterial cells and spores (cp. LUIT85 and references cited therein). The virus is preferably a DNA virus with a genome size of 2 kb to 10 kb base pairs, such as (but not limited to) the filamentous (Ff) phage M13, fd,

and f1 (inter alia see RASC86, BOEK80, BOEK82, DAYL88, GRAY81b, KUHN88, LOPE85, WEBS85, MARV75, MARV80, MOSE82, CRIS84, SMIT88a, SMIT88b) the IncN specific phage Ike and If1 (NAKA81, PEET85, PEET87, THOM83, THOM88a); IncP-specific Pseudomonas aeruginosa phage Pf1 (THOM83, THOM88a) and Pf3 (LUIT83, LUIT85, LUTI87, THOM88a); and the Xanthomonas oryzae phage Xf (THOM83, THOM88a). Filamentous phage are especially preferred.

Detailed Description Text (351):
Similar constructions could be made with other filamentous phage. Pf3 is a well known filamentous phage that infects Pseudomonas aerugenosa cells that harbor an IncP-1 plasmid. The entire genome has been sequenced (LUIT85) and the genetic signals involved in replication and assembly are known (LUIT87). The major coat protein of PF3 is unusual in having no signal peptide to direct its secretion. The sequence has charged residues ASP.sub.7, ARG.sub.37, LYS.sub.40, and PHE.sub.44 -- COO.sup.- which is consistent with the amino terminus being exposed. Thus, to cause an IPBD to appear on the surface of Pf3, we construct a tripartite gene comprising:

Detailed Description Text (388):
Among bacterial cells, the preferred genetic packages are Salmonella typhimurium, Bacillus subtilis, Pseudomonas aeruginosa, Vibrio cholerae, Klebsiella pneumonia, Neisseria gonorrhoeae, Neisseria meningitidis, Bacteroides nodosus, Moraxella bovis, and especially Escherichia coli. The potential binding mini-protein may be expressed as an insert in a chimeric bacterial outer surface protein (OSP). All bacteria exhibit proteins on their outer surfaces. Works on the localization of OSPs and the methods of determining their structure include: CALA90, HEIJ90, EHRM90, BENZ88a, BENZ88b, MAN088, BAKE87, RAND87, HANC87, HENR87, NAKA86b, MAN086, SILH85, TOMM85, NIKA84, LUGT83, and BECK83.

Detailed Description Text (401):
Other bacterial outer surface proteins, such as OmpA, OmpC, OmpF, PhoE, and pilin, may be used in place of LamB and its homologues. OmpA is of particular interest because it is very abundant and because homologues are known in a wide variety of gram-negative bacterial species. Baker et al. (BAKE87) review assembly of proteins into the outer membrane of E. coli and cite a topological model of OmpA (VOGE86) that predicts that residues 19-32, 62-73, 105-118, and 147-158 are exposed on the cell surface. Insertion of a ipbd encoding fragment at about codon 111 or at about codon 152 is likely to cause the IPBD to be displayed on the cell surface. Concerning OmpA, see also MACI88 and MAN088. Porin Protein F of Pseudomonas aeruginosa has been cloned and has sequence homology to OmpA of E. coli (DUCH88). Although this homology is not sufficient to allow prediction of surface-exposed residues on Porin Protein F, the methods used to determine the topological model of OmpA may be applied to Porin Protein F. Works related to use of OmpA as an OSP include BECK80 and MACI88.

Detailed Description Text (611):
10) Pseudomonas putida cytochrome P450.sub.CAM

Detailed Description Text (633):
5) Pseudomonas aeruginosa hemolysin

Detailed Description Text (1408):
DUCH88: Duchene, M, A Schweized, F Lottspeich, G Krauss, M Marget, K Vogel, B-U von Specht, and H Domdey, "Sequence and Transcriptional Start Site of the Pseudomonas aeruoinosa Outer Membrane Porin Protein F Gene", J Bacteriol (1987), 170:155-162.

Detailed Description Text (1551):
LUIT83: Luiten, RGM, JGG Schoenmakers, and RNH Konings, "The major coat protein gene of the filamentous Pseudomonas aeruoinosa phage Pf3: absence of an N-terminal leader signal sequence", Nucleic Acids Research (1983), 11(22)8073-85.

Detailed Description Text (1552):
LUIT85: Luiten, RGM, DG Putterman, JGG Schoenmakers, RNH Konings, and LA Day, "Nucleotide Sequence of the Genome of Pf3, an IncP-1 Plasmid-Specific Filamentous Bacteriophage of Pseudomonas aeruginosa", J Virology, (1985), 56(1)268-276.

Detailed Description Text (1718):
TRIA88: Trias, J, EY Rosenberg, and H Nikaido, "Specificity of the glucose channel formed by protein D1 of Pseudomonas aeruginosa", Biochim Biophys Acta (1988), 938:493-496.

CLAIMS:

11. The method of claim 9 wherein the initially chosen parental potential binding domain is selected from the group consisting of (a) binding domain of bovine pancreatic trypsin inhibitor, crambin, Cucurbita maxima trypsin inhibitor III, heatstable enterotoxin of Excherichia coli, .alpha. Conotoxin GI, .mu. Conotroxin GIII, .omega. Conotoxin GIV, apamin, charybdotoxin, secretory leukocyte protease inhibitor, cystatin, eglin, barley protease inhibitor, ovomucoid, T4 lysozyme, hen egg white lysozyme, ribonuclease, azurin, tumor necrosis factor, and CD4, and (b) domains at least substantially homologous with any of the foregoing domains which have a melting point of at least 50.degree. C.

15. The method of claim 9 wherein the initially chosen parental binding domain contains no more than 30 residues and at least 2 disulfides.

16. The method of claim 9 wherein the initially chosen parental binding domain contains no more than 60 residues and at least 3 disulfides.

17. The method of claim 9 wherein the initially chosen parental binding domain contains no more than 80 residues and at least 4 disulfides.

21. The method of claim 20 wherein the bacterial cell is selected from the group consisting of strains of Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumonia, Neisseria gonorrhoeae, and Bacillus subtilis.

24. The method of claim 22 wherein the chimeric potential binding protein substantially corresponds to the first 153 amino acids of LamB fused in frame to the potential binding domain.

32. The method of claim 29 wherein the second binding protein is produced by recombinant DNA techniques.

54. The method of claim 53 wherein the crosslink is a disulfide bond and the the amino acids at the first and second amino acid positions are cysteines.

55. The method of claim 54 in which the mini-protein domain has a single disulfide bond and the span of the bond is not more than nine amino acid residues.

56. The method of claim 54 wherein the mini-protein domain has a disulfide bond which bridges a sequence of amino acids which under affinity separation conditions collectively assume a hairpin supersecondary structure.

58. The method of claim 54 wherein the mini-protein domain comprises a plurality of intrachain disulfide bonds.

60. The method of claim 58 wherein the mini-protein domain has two disulfide bonds having a connectivity pattern of 1-3, 2-4.

62. The method of claim 58 wherein the mini-protein domain has three disulfide

bonds having a connectivity patter of 1-4, 2-5, 3-6.

☐ 24. Document ID: US 4943678 A

L11: Entry 24 of 25 File: USPT Jul 24, 1990

DOCUMENT-IDENTIFIER: US 4943678 A
TITLE: Pesticidal composition

Brief Summary Paragraph Table (2):
TABLE 1

Pest	Active ingredient	Citation
Erwinia carotovora	phenyl-alanine,	Gupta, Tripathi
var. atroseptica	ammonialiase, poly-	Indian J.E. 1976.
phenol 4. [3.]	streptomycin, Albergiana,	kanalicin, neomycin,
Phytopath. Zeit.	erithromycin, 81. [2.]	chloramphenicol Fusarium
Benomyl Leach, 1975	USDA 17. Jellis,	Taylor Ann. Appl.
Biol.	thiabendazol Munzel,	Bayer Land. 1976. 52.
[2.] Erwinia	MBC + chloramphenicol,	Ratba, Spez.
carotovora var.	Pflsch. 1977. 1. atr. +	Fusarium benomyl +
chloram-	Burth, Jahn	phenicol, Zineb +
Akad. Landw. Wiss. +	chloramphenicol [140]	Zineb + chloram-
Anonymus, Gops.	phenicol Soil. 1975. 30.	[1.] Erwinia +
antibiotic +	Sahurai, 1975. Pseudomonas	fungicide J.
Antibiotics, 29. [11.]		

Brief Summary Paragraph Table (3):
TABLE 2

Antibacterial effect of nitrofurane derivatives on axenial cultures MIC mg/ml minimal inhibitory concentration

Bacterium strain	819	1237	1552	2011	2013
Agrobacterium tumefaciens 0	4-8	1000	8-16	A	A
Agrobacterium tumef. C-58	4-8	1000	8-16	A	A
Agrobacterium tumef. B 6	4-8	1000	8-16	A	A
Agrobacterium rad.bac. K-84	2-4	1000	4-8	A	A
Erwinia herbicola D 5	1000	A	1000	A	A
Erwinia Uredovora	0.2-05	A	2-4	A	A
Erwinia atroseptica	4-8	A	8-10	A	A
Erwinia carotovora	4-8	A	8-10	A	A
Pseudomonas lachrymans	8-16	A	30-60	A	A
Pseudomonas mors-prunorum	1000	A	1000	A	A
Pseudomonas phaseolicola	30-60	A	60-120	A	A
Pseudomonas pisi	250-500	A	1000	A	A
Pseudomonas fluorescens	1000	A	1000	A	A
Rhizobium japonicum	8-16	A	30-60	A	A
Xanthomonas alfalfae	30-60	A	60- 120	A	A
Xanthomonas campestries	1000	A	1000	A	A
Xanthomonas fus.pv.phaseoli	30-60	A	60-120	A	A
Xanthomonas pelragonii	30-60	A	60-120	A	A
Xanthomonas vesicatoria	1000	A	1000	A	A
Xanthomonas orisae					
Corynebacterium fascians	15-30	A	60-120	A	A
Corynebacterium flaccumfac.	30-60	A	60-120	A	A
Corynebacterium michiganense	4-8	A	8-16	A	A
Corynebacterium nebraskense	4-8	A	8-16	A	A
Corynebacterium oortii	60-120	A	250-500	A	A

A = >1000 mg/ml

CLAIMS:

1. A method of combating phytopathogenic bacteria selected from the group consisting of Agrobacteria, Erwinia, Xanthomonas, Pseudomonas, and Corynebacterium, which comprises the step of applying onto a plant, the soil, or the environment thereof, an antibacterial composition, comprising a phytopathogenic bactericidally

effective amount of the compound of the Formula (I) ##STR5## wherein X is the group of the Formula (a) ##STR6##

or the Formula (b) ##STR7## in combination with an agriculturally acceptable inert carrier.

2. The method of combating phytopathogenic bacteria defined in claim 1 wherein the antibacterial composition further comprises a fungicide selected from the group consisting of:

1-butyl-carbamoyl-benzimidazole-2-methyl-carbamate;

2-methoxycarbonylamino-benzimidazole;

1,2-bis-(3-methoxycarbonyl-thioureido)-benzene;

2-(4-thiazolyl)-benzimidazole;

zinc-ethylene-bis-dithiocarbamate;

manganese-ethylene-bis-dithiocarbamate;

manganese and zinc ethylene-bis-dithiocarbamate;

tetramethyl-thiuram-disulfide; and

3-trichloromethylthio-tetrahydro-phthalimide,

wherein the ratio of the antibacterial compound of the Formula (I) to the fungicide is 1:99 to 99:1.

☐ 25. Document ID: US 4871530 A

L11: Entry 25 of 25 File: USPT Oct 3, 1989

DOCUMENT-IDENTIFIER: US 4871530 A
TITLE: Aqueous delayed-foaming cosmetic composition for hair and skin treatment

Brief Summary Text (32):
the biopolymer S88 generated by the strain Pseudomonas ATCC 31554 which comprises rhamnose, glucose, mannose and glucuronic acid in its structure; this biopolymer is described in British Patent No. 2,058,106;

Brief Summary Text (34):
the biopolymer S139 generated by the strain Pseudomonas ATCC 31644 which comprises rhamnose, glucose, mannose, galactose and galacturonic acid in its molecule; this biopolymer is described in particular in U.S. Pat. No. 4,454,316;

CLAIMS:

4. A composition according to claim 3 wherein the heterobiopolysaccharide is a

xanthane gum having a molecular weight of from 1,000,000 to 50,000,000 or biopolymer biopolymer PS87 which comprises glucose, galactose, mannose, fructose and glucuronic glucuronic acid units in its structure produced by Bacillus polymyxa, biopolymer S88 S88 produced by the AZTCC 31554 strain of Pseudomonas, biopolymer S130 produced by the strain Alcaligenes ATCC 31555, biopolymer S 198 comprising rhamnose, glucose, mannose, and glucuronic acid units in its structure produced by the strain Alcaligenes ATCC 31853, biopolymer S 139 comprising rhamnose, glucose, mannose, galactose and galacturonic acid units in its structure produced by the strain Pseudomonas ATCC 31644 or an exocellular biopolymer produced by the gram-positive or or negative species of bacteria, yeast or fungi.

24. A composition according to claim 19 wherein the cationic polymer is:

(1) a quaternized or unquaternized vinylpyrrolidone/dialkylaminoalkyl acrylate or methacrylate copolymer;

(2) a cellulose ether derivative comprising quaternary ammonium groups;

(b 3) a cellulose or cellulose derivative copolymer grafted with a water-soluble quaternary ammonium monomer;

(4) a cationic polysaccharide;

(5) a polymer comprising piperazinyl repeat units and divalent alkylene or hydroxyalkylene groups with straight or branched chains optionally interrupted by oxygen, sulphur or nitrogen or by aromatic or heterocyclic rings, or an oxidation and/or quaternization product of these polymers;

(6) a water-soluble polyaminopolyamide which is a polycondensate of an acidic compound with a polyamine, optionally crosslinked with an epihalohydrin, a diepoxide, a dianhydride, an unsaturated anhydride, a bis-unsaturated derivative, a bishalohydrin, a bisazetidinium, a bishaloacyldiamine or an alkyl bishalide or again with an oligomer resulting from the reaction of a difunctional compound reactive towards a bishalohydrin, a bisazetidinium, a bishaloacyldiamine, an alkyl bishalide, an epihalohydrin, a diepoxide or a bis-unsaturated derivative, the crosslinking agent being employed in an amount of from 0.025 to 0.35 mole per amine group of the polyaminopolyamine, or an alkylated or quaternized derivative thereof;

(7) a polyaminopolyamide derivative produced from the condensation of a polyalkylenepolyamine with a polycarboxylic acid followed by an alkylation using a difunctional agent;

(8) a product of the reaction of a polyalkylenepolyamine containing two primary amine groups and at least one secondary amine group with a dicarboxylic acid or a saturated aliphatic dicarboxylic acid containing from 3 to 8 carbon atoms, the molar ratio between the polyalkylenepolyamine and the dicarboxylic acid being from 0.8:1 to 1.4:1, the thus produced polyamino amide subsequently being reacted with epichlorohydrin in a molar ratio of epichlorohydrin to the secondary amine groups in the polyaminoamide of from 0.5:1 to 1.8:1;

(9) a cyclopolymer which is a homopolymer comprising repeat units of formulae (IX) or (IX') ##STR37## in which 1 and t are 0 to 1 and the sum 1+t is 1, R.sub.12 is hydrogen or a methyl group, R.sub.10 and R.sub.11 are each, independently of each other, an alkyl group containing from 1 to 22 carbon atoms, a hydroxyalkyl group or a lower amidoalkyl group, R.sub.10 and R.sub.11, together with the nitrogen atom to which they are attached, are a heterocyclic group, and Y.sup..crclbar. is an anion, or a copolymer comprising units of formula (IX) or (IX') and units derived from acrylamide or diacetoneacrylamide;

(10) a quaternary ammonium polymer comprising recurrent repeat units of formula: ##STR38## in which R.sub.13, R.sub.14, R.sub.15 and R.sub.16 are each, independently independently of each other, an aliphatic, alicyclic or arylaliphatic group containing from 1 to 20 carbon atoms or a lower aliphatic hydroxyalkyl group; or R.sub.13 and R.sub.14 and R.sub.15 and R.sub.16 together or separately form, with the nitrogen atoms to which they are attached, a heterocyclic ring which optionally contains a second heteroatom other than nitrogen; or R.sub.13, R.sub.14, R.sub.15 and R.sub.16 are each, independently of each other, a linear or branched C.sub.2 - C.sub.6 alkyl group substituted by a nitrile, ester, acyl, amide or ##STR39## in which R.sub.17 is an alkylene group and D is a quaternary ammonium group and A.sub.2 A.sub.2 and B.sub.2 are each, independently of each other, a polymethylenic group containing from 2 to 20 carbon atoms which is linear or branched, saturated or unsaturated and which optionally contains, linked to or inserted into the main chain, one or more aromatic rings or one or more oxygen or sulphur atoms or SO, SO.sub.2, disulphide, amino, alkylamino, hydroxyl, quaternary ammonium, ureido, amide or ester groups; or

A.sub.2 and R.sub.13 and R.sub.15 may form, with the two nitrogen atoms to which they are attached, a piperazine ring; and when A.sub.2 is a linear or branched, saturated or unsaturated alkylene or hydroxyalkylene radical, B.sub.2 may also be a group of formula:

--(CH.sub.2).sub.n --CO--D--OC--(CH.sub.2).sub.n--

in which D is

(a) a glycol residue of formula --O--Z--O-- in which Z is a linear or branched hydrocarbon group or a group of formula: ##STR40## in which x or y is an integer from 1 to 4 representing a specified and unique degree of polymerization or any number from 1 to 4 representing an average degree of polymerization; or

(b) a residue of a bis-secondary diamine;

(c) a residue of a bis-primary diamine of formula:

--NH--Y--NH--

in which Y is a linear or branched hydrocarbon group or a divalent group of formula:

--CH.sub.2 --CH.sub.2 --S--S--CH.sub.2 --CH.sub.2

(d) a ureylene group of formula:

--NH--CO--NH--

(11) a quaternary polyammonium polymer comprising units of formula: ##STR41## in which R.sub.18, R.sub.19, R.sub.20 and R.sub.21, are each, independently of each other, hydrogen or a methyl, ethyl, propyl, .beta.-hydroxyethyl, .beta.-hydroxypropyl or --CH.sub.2 CH.sub.2 (OCH.sub.2 CH.sub.2).sub.p OH group in which p is an integer of from 0 to 6, with the proviso that R.sub.18, R.sub.19, R.sub.20 and R.sub.21 are not simultaneously hydrogen, x and y are each, independently of each other, integers of from 1 to 6, m is an integer of from 0 to 34, X is a halogen and A is the residue of a dihalide;

(12) a homopolymer or copolymer derived from acrylic or methacrylic acid comprising units of formula: ##STR42## in which R.sub.24 is hydrogen or a methyl group, A.sub.1 is a linear or branched alkyl group containing from 1 to 6 carbon atoms or a hydroxyalkyl group containing 1 to 4 carbon atoms, R.sub.25, R.sub.26 and R.sub.27 are each, independently of each other, an alkyl group containing from 1 to

18 carbon atoms or a benzyl group, R.sub.22 and R.sub.23 are each, independently of each other, hydrogen or an alkyl group containing from 1 to 6 carbon atoms, and X.sub.2.sup..crclbar. is a methosulphate anion or a halide anion;

(13) a quaternary vinylpyrrolidone or vinylimidazole polymer; or

(14) a polyalkyleneimine, vinylpyridine or vinylpyridinium polymer, a condensate of a polyamine with epichlorohydrin, a quaternary polyureylene or a chitin derivative.

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L11: Entry 1 of 25 File: USPT Dec 10, 2002

DOCUMENT-IDENTIFIER: US 6492498 B1
**** See image for Certificate of Correction ****
TITLE: Multimeric immunotoxins

Brief Summary Text (7):
In addition, the invention encompasses a multimeric immunotoxic protein containing at least two fusion protein monomers, each of which includes a targeting domain and a toxic domain and is physically associated with the other fusion protein monomers. The targeting domain in all the multimeric immunotoxic proteins of the invention have significant binding affinity for a target molecule on a target cell. The fusion protein monomers can contain one or more coupling moieties and the physical association of the fusion protein monomer to one or more other fusion protein monomers can be mediated by at least one of the coupling moieties. The coupling moiety can be a terminal moiety, i.e., a C-terminal moiety or a N-terminal moiety. A coupling moiety can be, for example, a cysteine residue. Furthermore the coupling moieties can be heterologous coupling moieties. The fusion protein monomers in a particular multimeric immunotoxic protein can have the same amino acid sequence or different amino acid sequences. Targeting domains can be antibody fragments, e.g., single chain Fv and can have significant binding affinity for a target molecules on a T cell, e.g., a CD3 polypeptide. Alternatively, the targeting domain can be, for example, a polypeptide such as a cytokine, a ligand for a cell adhesion receptor, a ligand for a signal transduction receptor, a hormone, a molecule that binds to a death domain family molecule (e.g., Fas ligand, TNF-alpha, or TWEAK), an antigen, or a functional fragment of any of these polypeptides. The toxic domain can be, for example, any of the following toxic polypeptides: ricin, Pseudomonas exotoxin (PE), bryodin, gelonin, .alpha.-sarcin, aspergillin, restrictocin, angiogenin, saporin, abrin, pokeweed antiviral protein (PAP), or a functional fragment of any of these toxic polypeptides. The toxic domain can also be diphtheria toxin (DT) or a functional fragment thereof, e.g., a fragment containing amino acid residues 1-389 of DT. The target cell to which the multimeric immunotoxic proteins of the invention bind can be in a mammal. The mammal can be one suspected of having graft-versus-host disease (GVHD). A target cell to which the multimeric immunotoxic proteins bind can be a cancer cell, e.g., a neural tissue cancer cell, a melanoma cell, a breast cancer cell, a lung cancer cell, a gastrointestinal cancer cell, an ovarian cancer cell, a testicular cancer cell, a lung cancer cell, a prostate cancer cell, a cervical cancer cell, a bladder cancer cell, a vaginal cancer cell, a liver cancer cell, a renal cancer cell, a bone cancer cell, and a vascular tissue cancer cell.

Detailed Description Text (22):
Toxic domains useful in the invention can be any toxic polypeptide that mediates a cytotoxic effect on a cell. Preferred toxic polypeptides include ribosome inactivating proteins, e.g., plant toxins such as an A chain toxin (e.g., ricin A chain), saporin, bryodin, gelonin, abrin, or pokeweed antiviral protein (PAP), fungal toxins such as .alpha.-sarcin, aspergillin, or restrictocin, bacterial toxins such as DT or Pseudomonas exotoxin A, or a ribonuclease such as placental ribonuclease or angiogenin. As with the targeting domains, the invention includes the use of functional fragments of any of the polypeptides. Furthermore, a particular toxic domain can include one or more (e.g., 2, 3, 4, or 6) of the toxins or functional fragments of the toxins. In addition, more than one functional

fragment (e.g. 2, 3, 4, 6, 8, 10, 15, or 20) of one or more (e.g., 2, 3, 4, or 6) toxins can be included in the toxic domain. Where repeats are included, they can be immediately adjacent to each other, separated by one or more targeting fragments, or or separated by a linker peptide as described above.

CLAIMS:

1. A fusion protein molecule comprising a toxic domain, a targeting domain, and at least one heterologous coupling moiety, wherein cysteine residues forming disulfide bonds within said fusion protein are: (i) cysteine residues native to the toxic domain and form disulfide bonds within the toxic domain; or (ii) cysteine residues native to the targeting domain and form disulfide bonds within the targeting domain, domain, and wherein the at least one heterologous coupling moiety is a moiety through which a second fusion protein molecule can be bound to the fusion protein molecule.

2. A multimeric immunotoxic protein comprising at least two fusion protein monomers, wherein each fusion protein monomer: comprises a targeting domain and a toxic domain; and is physically associated with the other fusion protein monomers, wherein said targeting domain binds to a target molecule on a target cell, and if a targeting domain is an antibody fragment, said antibody fragment has fewer than fourteen immunoglobulin heavy chain constant region amino acid residues, wherein an antibody fragment with no immunoglobulin heavy chain constant region amino acid residues has one VH chain and one VL chain.

3. The multimeric immunotoxic protein of claim 2, wherein each of said fusion protein monomers further comprises one or more coupling moieties and the physical association of the fusion protein monomer is by at least one of the one or more coupling moieties.

8. The multimeric immunotoxic protein of claim 2, wherein each of the fusion protein monomers comprises the same amino acid sequence.

14. The multimeric immunotoxic protein of claim 2, wherein the toxic domain is a toxic polypeptide selected from the group consisting of: (a) ricin, (b) Pseudomonas exotoxin (PE); (c) bryodin; (d) gelonin; (e) .alpha.-sarcin; (f) aspergillin; (g) restrictocin; (h) angiogenin; (i) saporin; (j) abrin; (k) pokeweed antiviral protein (PAP); and (l) a functional fragment of any of (a)-(k).

22. A multimeric immunotoxic protein comprising at least two fusion protein molecules of claim 1, each fusion protein molecule being bound by at least one of the heterologous coupling moieties to one or more of other said fusion protein molecules.

L11: Entry 16 of 25 File: USPT Aug 24, 1999

US-PAT-NO: 5942602
DOCUMENT-IDENTIFIER: US 5942602 A

TITLE: Growth factor receptor antibodies

DATE-ISSUED: August 24, 1999

INVENTOR-INFORMATION:

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US-CL-CURRENT: 530/388.22; 424/1.49, 424/178.1, 424/9.34, 530/387.3, 530/388.8, 530/388.85, 530/391.3, 530/391.7, 536/23.1, 536/24.31

CLAIMS:

We claim:

1. A single chain polypeptide having a binding affinity for an epidermal growth factor receptor, said polypeptide comprising:

(1) a first polypeptide segment comprising the binding portion of the heavy chain variable domain of monoclonal antibody 14E1; and

(2) a second polypeptide segment comprising the binding portion of the light chain variable domain of monoclonal antibody 14E1.

2. A single chain polypeptide of claim 1, further comprising a polypeptide linker joining the heavy chain variable domain to the light chain variable domain.

3. A single chain polypeptide of claim 1, wherein the polypeptide linker is 15 amino acids.

4. A single chain polypeptide of claim 1, wherein the heavy chain variable domain is positioned amino-terminal to the light chain variable domain.

5. A single chain polypeptide of claim 1, further comprising a biologically-active component.

6. A single chain polypeptide of claim 1, wherein the single chain polypeptide further comprises a third polypeptide segment comprising a biologically-active component.

7. A single chain polypeptide of claim 5, wherein the biologically-active component is cytotoxic or cytostatic.

8. A single chain polypeptide of claim 7, wherein the biologically-active component is a cytotoxin.

9. A single chain polypeptide of claim 1, wherein the single chain polypeptide further comprises a third polypeptide segment comprising a cytotoxic effective portion of Pseudomonas exotoxin A.

10. A single chain polypeptide of claim 1, further comprising a third polypeptide segment comprising a cytotoxic or cytostatic effective portion of biologically-active component.

11. A single chain polypeptide of claim 1, which is scFv(14E1) or scFv(14E1)-ETA.

12. A single chain polypeptide of claim 1, further comprising a radiodiagnostic or radio-therapeutic agent.

13. A single chain polypeptide of claim 1, further comprising a MRI contrast agent.

14. A single chain polypeptide of claim 1, wherein the binding portion of the heavy chain variable domain comprises the sequences, SEQ ID NOs: 3,4,5 and 6, of monoclonal antibody 14E1 and the binding portion of the light chain variable domain comprises the sequences, SEQ ID NOs: 1 and 2, of the light chain variable domain of antibody 14E1.

15. A nucleic acid comprising a nucleotide sequence coding for a single chain polypeptide having a binding affinity for an epidermal growth factor receptor, said nucleic acid comprising:

(1) a first nucleotide sequence coding for a first polypeptide segment comprising the binding portion of the heavy chain variable domain of antibody 14E1; and

(2) a second nucleotide sequence coding for a second polypeptide segment comprising the binding portion of the light chain variable domain of antibody 14E1.

16. A nucleic acid of claim 15, further comprising a third nucleotide sequence coding for a polypeptide linker positioning the heavy chain variable domain 5' to the light chain variable domain by a polypeptide.

17. A nucleic acid of claim 15, further comprising a third nucleotide sequence coding for a biologically-active component.

18. A nucleic acid of claim 15, wherein the biologically-active component is a cytotoxic effective portion of Pseudomonas exotoxin A.

19. A nucleic acid of claim 15 which codes for scFv(14E1) or scFv(14E1)-ETA.

20. A nucleic acid of claim 15 which is pSW50-14E1 or pSW202-14E1.

21. A method of imaging cells expressing an epidermal growth factor receptor comprising

administering an effective amount of a compound of claim 1, wherein the single chain polypeptide comprises a radiolabeled compound or a MRI contrast agent.

22. A method of claim 21, wherein the single chain antibody is scFv(14E1).

23. A method of claim 21, wherein the cell are a breast cell carcinoma.

24. A double chain polypeptide having a binding affinity for an epidermal growth factor receptor, said polypeptide comprising:

(1) a first polypeptide segment comprising the binding portion of the heavy chain variable domain of monoclonal antibody 14E1; and

(2) a second polypeptide segment comprising the binding portion of the light chain variable domain of monoclonal antibody 14E1,

wherein the heavy chain variable domain is joined to the light chain variable domain by a covalent linkage extending from each of the domains.

25. A double chain polypeptide of claim 24, wherein the covalent linkage is a disulfide bond.